



PHD

## Autologous mixed lymphocyte reaction in myasthenia gravis

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AUTOLOGOUS MIXED LYMPHOCYTE REACTION IN MYASTHENIA GRAVIS

Submitted by Ian Richards  
for the degree of PhD  
of the University of Bath  
1987

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## ABBREVIATIONS

AChR	AcetylCholine Receptors
AET	2-aminoethylisothiuronium bromide hydrobromide
AMLR	Autologous Mixed Lymphocyte Reaction
BSS	Balanced Salt Solution
CFA	Complete Freund's Adjuvant
Con-A	Concanavalin-A
EAMG	Experimental autoimmune myasthenia gravis
E.P.+N	Endogenous Peroxidase positive cells plus null cells
ERFC	Erythrocyte-rosette forming cells
FCS	Foetal calf serum
GAM	Goat anti-mouse antibody
HLA	Human Leucocyte Antigen
Ia	Immune-associated (antigen)
IFA	Incomplete Freund's Adjuvant
IL-2	Interleukin 2
Ir	Immune response (genes)
LPS	Lipopolysaccharide
mepp's	miniature end-plate potentials
MG	myasthenia gravis
MHC	Major Histocompatibility Complex
ORBC	Ox red blood cells
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PHA-P	Phytohaemagglutinin-P

PWM	Pokeweed mitogen
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
Smlg +ve	Surface membrane immunoglobulin positive cells
SRBC	Sheep red blood cells

## SUMMARY

The autologous mixed lymphocyte reaction was studied in normal individuals, in hospital out-patients suffering from the autoimmune disorder myasthenia gravis (MG) and to a limited extent in patients with rheumatoid arthritis (RA).

The results of a comprehensive study indicated that normal individuals' responses may fall into one of three groups - high, medium and low responses. The AMLR response was shown to be reproducible on repeat assay for any responder type, although individual response variation within a group may be relatively high. No correlation between the normal AMLR and the normal response to the mitogens concanavalin-A (Con-A), or phytohaemagglutinin-P (PHA-P) was found.

The AMLR response was shown to be statistically significantly depressed in patients with MG ( $P < 0.0001$ ) and in patients with RA ( $P < 0.0001$ ), when compared to the responses of healthy control individuals. No differences were found between the kinetic AMLR responses of normal individuals and those of MG patients or RA patients. The proliferative responses to the mitogens PHA-P and Con-A of myasthenic lymphocytes ( $P < 0.0001$  and  $P < 0.006$  respectively) and of rheumatoid



lymphocytes ( $P < 0.0001$  and  $P < 0.001$  respectively) were also found to be statistically significantly depressed in comparison to normal responses.

In an attempt to determine the cellular defects that may occur in the autoimmune process, the animal model of MG, experimental autoimmune myasthenia gravis (EAMG), was induced and maintained in New Zealand white rabbits by using purified acetylcholine receptor from the electric ray, Torpedo marmorata.

The AMLR responses of the rabbits with EAMG were statistically significantly decreased ( $P < 0.04$ ) in comparison to normal rabbit AMLR. No differences were found between pre- and post-immunisation mitogen responses in the test rabbits.

The rabbit model of EAMG is not considered to be a useful model for studying cellular immunoregulation because of the low normal rabbit cellular reactivity.

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## 1. INTRODUCTION

"the organism has contrivances by means of which the immunity reaction, so easily produced by all kinds of cells, is prevented from reacting against the organism's own elements and so give rise to autotoxins .... Only when the internal regulating contrivances are no longer intact can great dangers arise."

Paul Ehrlich (1901).

Historically, immunologists have taken the view that the specificity of an immune response is the most distinguishing and important feature of the system. Thus, disorders of the immune system can be considered in terms of alterations in specificity, and there should be a specific antibody or cell-mediated immune response that is heightened or decreased to account for the disease process.

The immune system is a complex network of interactions whose internal regulation requires self-recognition. Regulation may involve cells, antibodies, amplification systems (e.g. complement) and combinations of these elements.

## 1.1. Controlled self-reactivity (tolerance to self-antigens) in the normal individual

### 1.1.1 Clonal deletion

The view that the immune system is in a constant state of self recognition has only recently evolved. Early concepts required that an organism could not react against its own antigenic determinants and Burnet (1959) proposed that natural self tolerance, the absence of reactivity to self-antigens, required either the absence of lymphocytes with receptors for self or the inaccessibility to lymphocytes of self-antigens.

Autoimmunity was thought to result either from the development, by somatic mutations, of self-reactive lymphocytes or from contact of lymphocytes with normally inaccessible self antigens (Burnet, 1959). This hypothesis received enthusiastic support and remains a possible mechanism for the loss of tolerance. However, more recently, the existence of self-reactive lymphocytes has been demonstrated in normal healthy animals and humans (Bankhurst et al, 1973; Baxl y et al, 1973; Cohen and Wekerle, 1973). Elimination of particular clones of

lymphocytes may nevertheless have a role, together with other self-tolerising processes, as no lymphocytes have been found with receptors for albumin, which circulates freely in high concentration in the peripheral blood (Nossal et al, 1973).

#### 1.1.2 The role of blocking factors

There is much logic in the notion that non-immunogenic self-antigens act to block potentially self-recognising lymphocytes. The constant turnover and shedding of normal cell membrane components would seem to provide a constant source of soluble fragments of self-antigens to compete with immunogenic membrane bound forms of these antigens (Doljanski, 1973). Indeed, only after significant tissue trauma would sufficient quantities of immunogenic membrane-bound antigens be released and provoke the afferent sensitising stage of an anti-self immune response. Also, the concentration of blocking self-antigens would be highest near those cells which bear the respective immunogenic self-antigens. Thus, negative feedback blocking would be ensured to be most effective at the critical point when a

potentially self-recognising lymphocyte approaches the cell (Baxley et al, 1973).

#### 1.1.3 Effects of immune complexes

Immune complexes might be expected to form as soon as autoantibodies are secreted and act as a further line of defence against autoimmunity. Such complexes, soluble because of the relative excess of self-antigens, may block cytotoxic effects [as they may do in the case of tumour cells (Baldwin et al, 1972)], and the production of autoantibodies by B cells. They may also induce the generation of T suppressor cells (Gorczynski et al, 1974), adding a positive regulatory control to the peripheral blocking of autoreactivity.

#### 1.1.4 Antigen concentration dependence

Tolerisation of T and B cells has been shown to be dependent on the concentration of antigen present (Chiller et al, 1971); T cells can be readily tolerised at low concentrations of antigen. Thus, although B cells may remain immunocompetent they are prevented from producing antibody by the lack of help from the tolerised T cells. The experiments of Triplett, (1962)



showing adult rejection of the frog hypophysis removed at the larval stage would argue for a requirement for the persistence of self-antigen in order to maintain tolerance. However, Rose (1983) showed that unoperated control animals were as responsive to native thyroglobulin as were thyroidectomised animals. Thus, tolerance to some self-antigens is maintained even when the antigen is removed whereas tolerance to other self-antigens may cease.

#### 1.1.5 Sequestration of self-antigens

Sequestered self-antigens such as those of the testes, brain and lens of the eye are not normally in contact with circulating lymphocytes. However, trauma or infection may expose these antigens to the immunocompetent cells, inducing an autoimmune response.

Binding and response to these antigens does not preclude the clonal deletion theory, as these antigens have presumably been sequestered throughout the life of the animal and deletion of the reactive cells in the neonate would not have occurred. Thus, sequestration of certain antigens may provide the tolerance to these antigens in the normal animal.

#### 1.1.6 The role of regulatory T cells

It is now well documented that specific or non-specific stimulation of the immune system generates a population of T lymphocytes with suppressor activities. Such suppressor T cells ( $T_s$ ) have been demonstrated following antigen priming (Tada et al, 1975), tolerance induction (Basten et al, 1975), and idiotype suppression (Eichmann, 1975).

Antigen-specific suppressive factors have been extracted from  $T_s$  cells, obtained from various sources (Tada et al, 1975; Tanaguchi and Miller, 1977). These are glycoproteins of molecular weight 35,000-50,000 and do not possess Ig determinants but do have a determinant coded for by the I-J region of the MHC. This factor can replace  $T_s$  cells both in vivo and in vitro (Tanaguchi and Miller, 1977).

In view of the findings presented above, the mechanisms underlying the establishment of self tolerance and the development of autoimmunity must be reassessed. Burnet proposed that tolerance results from clonal deletion of self-reactive clones in the neonate and that

autoimmunity results from the generation of self-reactive somatic mutations. It appears more likely that the induction and maintenance of tolerance arises from the  $T_s$  cell activity described above and that autoimmunity results from a breakdown in the control exerted by these cells, or by cells which induce the  $T_s$  activity.

Tolerance to self MHC antigens is learned during the intra-thymic differentiation of T cells (Miller, 1978; Zinkernagel, 1978). Tolerance to other self components and antigens for T and B cells is more likely to result from the active suppression by  $T_s$  rather than by the inflexible deletion of cells from a system which must encounter modified self antigens, previously sequestered self antigens and cross-reactive antigens.

#### 1.1.7 Idiotypic network

The network theory of the immune response proposed by Jerne (1974) suggests that antigen stimulates the formation of an antibody expressing an idiotype ( $Ab_1$ ) to which anti-idiotypic ( $Ab_2$ ) antibodies are generated.

Anti-idiotypic antibodies ( $Ab_2$ ) interfere not only with the production of additional

quantities of antibodies of the corresponding specificity but may also block antigen-binding sites on the surfaces of T lymphocytes. These idiotypic interactions may serve to protect the organism, but cross-reaction of certain idiotypes may lead to an autoimmune response (Cooke et al., 1983).

#### 1.1.8 Tolerance breakdown

A combination of autoantigen and exogenous antigen may form immunogenic units capable of stimulating T cell help that can activate the immunocompetent B cells to produce autoantibody (see Figure 1. ). Virus particles, bacteria and drugs may each provide the stimulus for this T cell help by either cross-reaction or by forming altered self-determinants on the surfaces of target cells. Cross reaction between antigens present on the myocardium and antigens present on group A  $\beta$ -haemolytic streptococci can lead to an autoimmune response directed against this tissue in susceptible individuals (Kaplan and Svec, 1964).

A possible mechanism for the development of autoimmunity by T cell bypass because of the generation of new carrier determinants or by

SELF TOLERANCENEW CARRIER

Tolerisation or clonal  
deletion of T helper/  
inducer cell

CROSS-REACTING: DRUG / VIRUS/  
ANTIGEN

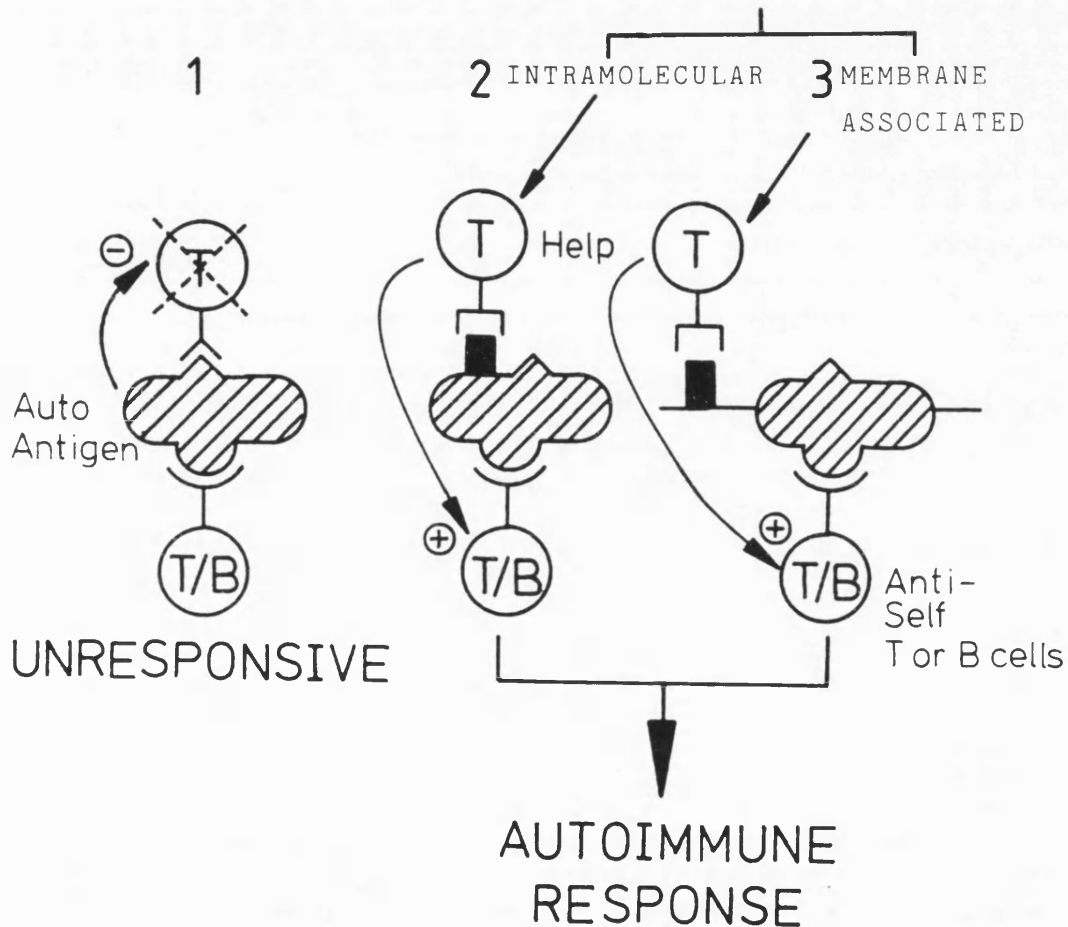


Figure 1. By-pass mechanisms for the induction of Autoimmunity.

1. Self-tolerant state: tolerisation, clonal deletion or suppression of the self-reactive T helper cell.
2. Provision of new carrier determinants through intramolecular cross reacting antigen, drug or virus particle.
3. Provision of new carrier determinants through membrane associated cross reacting antigen, drug or virus particle.

cross reaction may be represented by Figure 1. , as suggested by Cooke et al, (1983). If normal immunoregulatory circuits are intact, the autoantibody formed following stimulation of a T cell helper signal by self-antigen can be interrupted by suppressor T cells. Deficient suppressor T cell activity or a reduced number of these cells may allow the autoimmune response to proceed and result in the pathogenesis of an autoimmune disease.

#### 1.2. The autologous mixed lymphocyte reation (AMLR)

It is clear that, in certain cases, self-reactivity does occur in vivo . Smith and Steinberg, (1983), proposed that controlled self-reactivity represents a normal event and that self-reactions are important mechanisms of normal immune reactivity and immunoregulation. The autologous mixed lymphocyte reaction (AMLR) may represent one such mechanism. Quantitative abnormalities can be seen to account for disease development represented by the degree of stem cell proliferation, autoantibody generation or immune complex formation. Rather than being caused by a single factor, autoimmune diseases are now considered as being multi-factorial in aetiology. Individual genes, environmental

factors and hormonal balance may contribute to disease predisposition and development. Stimulation of or interference with normal immunoregulation coupled with an alteration in hormonal homeostasis may lead to manifestation of autoimmune disease.

Efficient disposal of foreign antigens from the body requires the participation of both T and B lymphocytes. It is known that this participation requires the activation of the T cells by antigen presented in association with a surface marker on non-T leucocytes and that the antigen presenting cell must bear Major Histocompatibility Complex (MHC) encoded antigens matching those of the responding cell.

It has been confirmed (Glimcher et al, 1980) that antigen alone cannot activate T helper ( $T_H$ ) cells and that a cell bearing specific surface markers must also co-operate. These surface markers or Immune associated (Ia) antigens have been shown in the murine system to be coded for by the immune response (Ir) genes (Rosenthal et al, 1973; Thomas et al, 1977; Benacerraf, 1981) which are products of the H-2 locus of chromosome 17. This locus is analogous to the HLA-D region on chromosome 6 in man.

Bain et al, (1964) and Bach and Hirschhorn (1964) observed that mixtures of lymphocytes from two genetically non-identical individuals are capable of stimulating each other in vitro (the mixed lymphocyte reaction). The subsequent genetic and immunological analysis of this phenomenon (Bach and Amos, 1967; Albertini and Bach, 1968; Amos and Bach, 1968; Zoschke and Bach, 1971) established the mixed lymphocyte reaction as a means of measuring the degree of histocompatibility at Ia loci between individuals and has provided an important tool for the study of immunobiology and genetics of tissue transplantation.

What is perhaps much more suprising is that Ia bearing cells can induce autologous T cell proliferation; a finding which indicates that the immune system contributes more than just a mechanism for the removal of foreign antigen from the body. This phenomenon has been termed the Autologous Mixed Lymphocyte Reaction (AMLR) and was first observed as a significant proliferative response in the background thymidine incorporation in the mixed lymphocyte reaction (Etheredge et al, 1973). Subsequently Opelz et al, (1975) demonstrated that the proliferative



response of unfractionated lymphocytes in culture could be dramatically enhanced by increasing the ratio of non-T cells to T cells. The AMLR displays the classical attributes of an immune response; memory and specificity (Weksler and Kozak, 1977; Sakane and Green, 1979).

#### 1.2.1 Responding and stimulating cell types

Much controversy has been generated over the exact nature of the stimulating and responding cells in the AMLR. The responding cells were found to be T lymphocytes and the stimulating cells non-T leucocytes (Opelz et al, 1975; Kuntz et al, 1976).

The techniques used to prepare T cells have identified characteristics of those T cells that proliferate in the AMLR. They are low density lymphocytes (Stober and Loehnen, 1978) that form early rosettes with sheep red blood cells (SRBC) (Yu, 1978) and express receptors for autologous erythrocytes (Fournier and Charriere, 1981). They react with the OKT4 monoclonal antibody (Kozak et al., 1981) and respond to Concanavalin-A (Con-A) (Sakane and Green, 1979). Indeed, 90% of autologous erythrocyte-rosetted T cells react with OKT4 monoclonal antibody whereas

only 60% of normal T cells react in this way.

Numerous marker antigens have been described to define various subpopulations of T cell lineage. These include the presence of receptors for immunoglobulin isotype Fc (Gupta, 1982), sensitivity of SRBC-T cell rosette formation to theophylline (Shore et al., 1978), and rosette formation between T cells and autologous RBC (Sandilands et al., 1974). These subpopulations have been suggested as forming different responding subsets with regard to the stimulating antigen. The proposed relationship between these cells may be seen in Figure 1.1., as suggested by Weksler et al., (1981).

Heterogeneity of autologous responding T cells was reported by Hausman et al., (1980), with regard to the nature of the responding and stimulating cell pairs. Hausman et al (1980), reported that one subset of human T cells, identified by the monoclonal antibody T29, were responsive to stimulation by macrophages that display the surface determinant recognised by the monoclonal antibody Mac120. T cell subsets not displaying the T29 marker i.e. T29<sup>-</sup>, were found to be stimulated by autologous B lymphocytes and null cells.

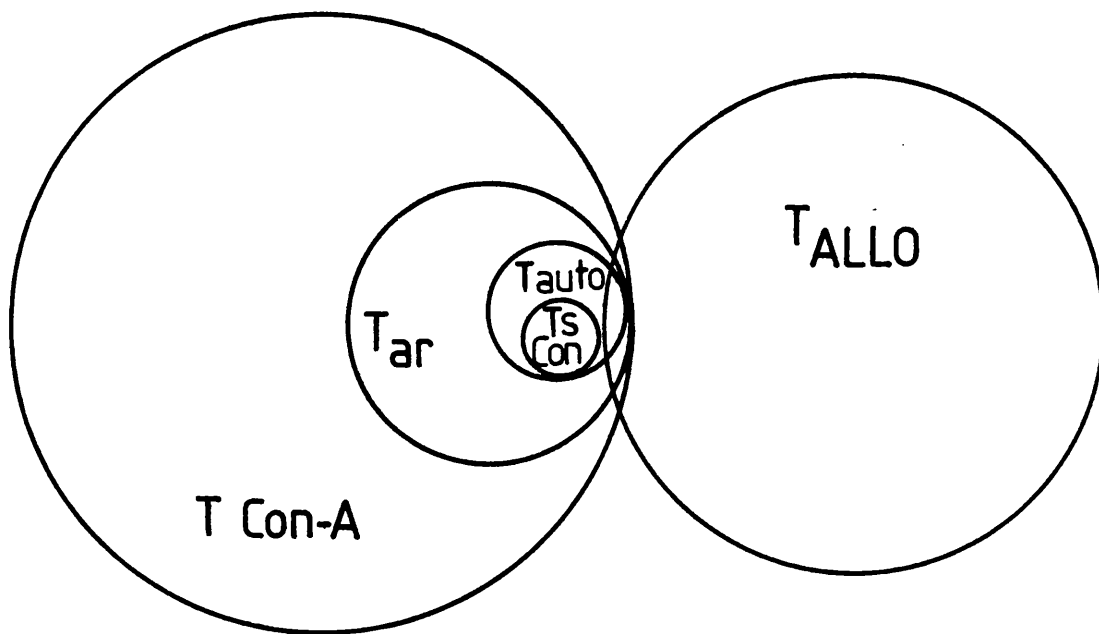
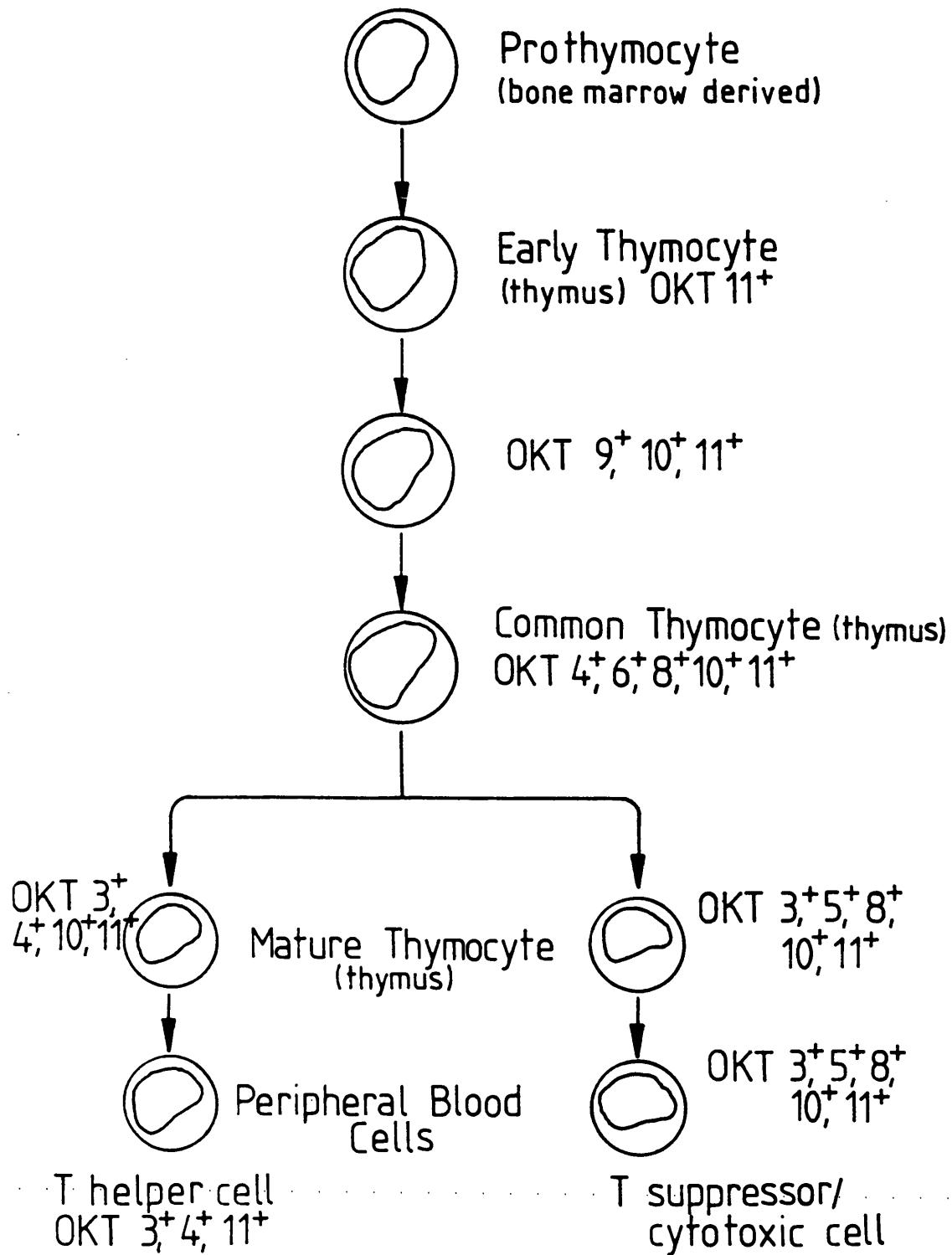


Figure 1.1. Relationship between ConA activated (T ConA) T cells, allogeneic cell activated (T allo) T cells, autologous cell activated (T auto) T cells, T cells that form rosettes with autologous erythrocytes (Tar) and T cells that develop suppressor activity in the presence of ConA (Ts Con). The area of the circles is not intended to reflect the sizes of the T cell subsets.

The OKT series of anti-(human lymphocyte) monoclonal antibodies have been used for the phenotypic identification of T cells as they progress through their maturational development and acquire different functions (Fig 1.2). OKT4 antibody reacts with a population of T cells ( $\text{OKT4}^+$ ) that is believed to define a subset containing helper/inducer functions for B cell Ig production, whereas OKT8 antibody reacts with a subset of T cells ( $\text{OKT8}^+$ ) that contains suppressor/cytotoxic functions (Reinherz and Schlossman, 1980). By using subset-specific antibodies and complement, various groups have shown that  $\text{OKT4}^+$  T cells are the major responders in the AMLR (Kozak et al., 1977; Gupta 1983). Auto-activated T cells, fractionated following a primary AMLR using a Percoll density gradient centrifugation procedure, were shown to react with OKT4 antibody at levels of 90%. However, only 10% of the same auto-activated cells reacted with the OKT8 antibody (Kozak et al, 1981).

The differential ability of  $\text{Mac120}^+$  and not  $\text{Mac120}^-$  macrophages to act as stimulators for  $\text{T29}^+$  cells could indicate expression of Ia antigen on  $\text{Mac120}^+$  but not on  $\text{Mac120}^-$  cells, or a

Figure 1.2. Maturation of functional T cells.



difference in the density or affinity of these antigens. The heterogeneous non-T cell population contains B cells, monocytes, null cells, dendritic cells and macrophages and various reports (Royston et al, 1974; Kuntz et al, 1976; Sakane et al, 1978; Gottlieb et al, 1979; Palacios and Moller, 1981) have suggested maximal proliferation caused by using one or a combination of the possible stimulators. Shen et al (1983), found that macrophages recognised by the monoclonal antibody OKM5<sup>+</sup>, but not OKM1<sup>+</sup>, were stimulatory in the AMLR, although no quantitative difference was found between OKM1<sup>+</sup> AND OKM5<sup>+</sup> macrophage Ia expression. Both Mac120<sup>-</sup> (Hausman et al, 1980) and OKM1<sup>+</sup> macrophages have been shown to be effective stimulators of an allogeneic MLR, indicating the presence of Ia antigens.

That Ia antigens play a central role in T cell activation in the AMLR was demonstrated by Mingari and Moretta (1982), when Ia positive but not Ia negative autologous peripheral blood mononuclear cells were shown to function as effective stimulating populations. Stimulating capacity is not restricted to the non-T cell population because Ia<sup>+</sup> (mixed lymphocyte culture or mitogen activated) T cells are equally

effective (Ferrone, 1980, Mann and Sharrow, 1980, Russo et al, 1981, Mingari and Moretta, 1982). Addition of two different monoclonal antibodies directed against Ia strongly inhibited cell proliferation in autologous mixed lymphocyte cultures.

Although it has been established that the major responding cell in the AMLR is the  $T_H$  ( $OKT4^+$ ) cell (Smolen et al, 1981), the  $OKT8^+$  subset, which exerts potent suppression of Ig synthesis, could exhibit substantial proliferation when exogenous Interleukin-2 (IL-2; T cell growth factor) is supplied 'in vitro' or when co-cultured in the presence of irradiated  $OKT4^+$  cells. Triggering of T cells by an Ia positive cell may result in the expression of IL-2 receptors. Any procedure that inhibits or blocks the expression of IL-2 receptors in T cells or causes a decreased production or availability of IL-2 would impair the proliferation of activated T cells. Equally, reduced expression and/or presentation of Ia by  $Ia^+$  cells will affect the activation and subsequent IL-2 responsiveness of T cells.

The degree of activation of responding cells in the AMLR has been shown to be greater than the

number of T cells induced to proliferate (Smolen et al, 1982). Also, analysis showed that a very small percentage of responder T cells (less than 1%) respond initially to signals from autologous non-T cells. There is a three day delay before substantial proliferation and activation takes place providing time for amplification and suppressive regulatory processes to occur.

#### 1.2.2 Effector functions of the AMLR

The secondary immunological consequences of T cell activation in human AMLR and in the murine analogue, the syngeneic mixed lymphocyte reaction (SMLR), are now known to be the induction of both T helper and suppressor cell functions although doubt remains over the demonstration of auto-cytotoxic T cell generation.

Several reports have failed to detect cytotoxicity in the AMLR (Vande Stouwe et al, 1977; Smith, 1978; Katz et al, 1978). However, Tomonari (1980) observed the generation, in the AMLR, of cytotoxic T cells ( $T_c$ ) having similar characteristics to natural killer (NK) cells. Maximal cytotoxicity was observed on day 8-9 of a



mixed lymphocyte culture and DNA synthesis and cell proliferation were required for  $T_c$  cell generation. These observations have since been supported by the findings of Goto and Zvaifler (1983) who described the AMLR  $T_c$  cell as expressing the PAN T cell marker 9.6 and the activated T cell marker 4F2 and closely resembling NK cells. The generation of immunoglobulin secreting cells (ISC) during the AMLR was initially described by Gatenby et al, (1982) and subsequently confirmed by Romain and Lipsky (1983). As with other AMLR effector functions, ISC activity could be augmented by foetal calf serum (FCS) in the culture medium.

#### 1.2.3 The role of the AMLR

The AMLR has been regarded for some time as little more than a laboratory curiosity. Several workers have suggested that the AMLR might result from perturbations of the responding or stimulating cells during cell fractionation or exposure to xenogeneic antigens in the separation procedures or during culture (Huber et al, 1982; Kagan and Choi, 1983; MacDermott and Bragdon, 1983) but these findings have been questioned. Moody et al, (1983) and Naides et al, (1985) have

used cell populations separated by passage over nylon wool to demonstrate that xenoantigen is not required for an AMLR although others have shown that xenoantigens may enhance the AMLR proliferative response of some, but not all, normal healthy individuals tested (Indiveri et al, 1985). When the AMLR was described by Kuntz et al (1976), a potential role for this reaction in the regulation of the immune response was suggested. Subsequent support and acknowledgement of the AMLR as a true immune phenomenon and evidence for its immunoregulatory role came with the demonstration of memory and specificity (Weksler and Kozak, 1977, Sakane and Green, 1979), of helper activity (Hausman and Stobo, 1979), of suppressor activity (Sakane and Green, 1979; Innes et al, 1979; Smith and Knowlton, 1979) and of cytotoxic activity (Tomonari, 1980). Soluble factors with immunoregulatory functions were also demonstrated in vitro (Yu et al, 1980; Lattime et al, 1981).

The impaired AMLR observed in many patients with diseases possibly associated with abnormal immunoregulation may lend additional support for an immunoregulatory role of the AMLR. Impaired AMLR in patients with autoimmune diseases (Smith and De Horatius, 1982; Sakane et al, 1978;

Miyasaka et al, 1980; Hafler et al, 1985; Richards et al, 1986), immunodeficiency states (Engleman et al, 1980; Gupta et al, 1984) and immunoproliferative disorders (Knight et al, 1983; Smith et al, 1977) have been demonstrated, although conflicting observations have been reported by other groups. Notably, increased AMLR have been reported in a group of patients with multiple sclerosis (Birnbaum and Kotilinek, 1981) and also in patients with myasthenia gravis (Greenberg et al, 1985), both autoimmune diseases. This latter report will be further explored in the Discussion section.

The recent findings of Kotani et al (1984), that a part of the AMLR may represent an important pathway for the activation of a feedback suppressor mechanism among T cells expressing the OKT4 marker, argues for a central role of the AMLR in antigen non-specific immunoregulation, in the normal, clinically healthy individual. When, for any reason, this immunoregulatory mechanism breaks down, the "horreur autotoxica" described by Ehrlich may be established and manifest itself as one of many different autoimmune states.

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### 1.3. Autoimmune diseases

If the immune system can be called into action to protect the host from potential environmental threats, then it must also be endowed with mechanisms to prevent an immunological attack on its own tissues. As discussed in section 1.1, self/non-self discrimination may be controlled via one or a combination of possible mechanisms.

Recent developments in autoimmunity suggest that there are three stages in response to self. These may be called autorecognition, autoimmunity and autoimmune disease. The first is now regarded as normal and fundamental to a network theory of immunological control that is based upon the recognition of idiotypes and antigens related to the MHC. Autoimmunity is, as the term implies, a state of controlled reactivity against self-antigens whereas autoimmune disease is the result of a breakdown in the mechanisms that maintain immunological homeostasis through autoimmunity.

Human autoimmune disease states may be broadly classified into a spectrum having

organ-specific disorders such as Hashimoto's thyroiditis at one end, and non-organ-specific disorders such as systemic lupus erythematosus (SLE) at the other. Organ specific disorders appear to involve immunological attack on a particular organ by virtue of a response directed against a target antigen restricted to that organ. Non-organ specific disorders involve lesions which are widespread throughout the body. A wide overlap may occur within diseases at each end of the spectrum. For example, patients with autoimmune thyroid disease may have a high incidence of gastric autoantibodies, whereas patients with rheumatological disorders tend to have a combination of rheumatoid arthritis with some features of SLE.

Many diseases that are immune-mediated are influenced to some extent by the genetic background of the individual and the prevailing state of the immune system. The abnormality that results in autoimmune disease expression may lie in the self-antigen, the immune process, or both. Thus, one may have an abnormal self-antigen with a normal immune response to it, an abnormal response to a normal self-antigen, or an abnormal response to an abnormal antigen.

The presence of auto-antibodies, or even their distribution in tissues, does not necessarily imply that the cause of lesions and of the clinical symptoms of the disease is the autoimmune process itself. However, the uncontrolled generation of anti-self antibodies or of auto-aggressive cells does infer a lack of immunological regulation.

Humoral responses may take the form of deposition of antibody-antigen complexes in the tissues, activating complement and resulting in inflammation (type II and type III hypersensitivity). Cell-mediated lesions may be caused by cytotoxic T cells or by antibody-dependent, cell-mediated cytotoxicity of killer cells and macrophages.

A comprehensive review of the AMLR in health and disease states in man by Gupta (1983), discussed the cellular responses in various autoimmune states, and the likely basis for the observed defects. The vast majority of the observations reviewed by Gupta (1983) and those up to the present date appear to support the notion that the AMLR is decreased in a variety of autoimmune diseases.

### 1.3.1 Myasthenia Gravis

### 1.3.2 Historical Aspects

One of the earliest observations of MG was recorded in 1683 in a book written by Dr Thomas Willis under the heading "The Habitual and Spurious Palsies". Many accounts of myasthenia have since been reported and are reviewed by Harrison and Behan (1986), the most comprehensive account is that of Campbell and Bramwell (1900). Some of the early observations are summarised below.

Wilks, (1877) described a female with weakness, an unexplained squint and difficulty in speaking (dysarthria). Death was due to respiratory paralysis. Erb, (1879) reported three patients with drooping of both eyelids (bilateral ptosis), and weakness of neck muscles. Fluctuations in severity and the apparent recovery of 2/3 patients argued against progressive bulbar palsy. Goldflam, (1893) found neuromuscular weakness without muscular atrophy in several patients. Good sphincter control and normal palatal reflex differentiated 'Erb-Goldflam complex' from true bulbar palsy.

Jolly, (1895) described 'Myasthenia gravis pseudoparalytica' in two young boys with decremental response of muscles to tetanic indirect electrical stimulation. The term 'myasthenia gravis' was generally adopted in 1899. Bell, (1917) and Holmes, (1923) both found association of thymic tumours with MG.

Following the account of Campbell and Bramwell (1900) in which ocular and vocal muscle involvement was commented on, independent conclusions were drawn with regard to the fact that the prime site of disease in MG was the muscle (Weigert, 1901; Herzog, 1917; Marburg, 1931; Norris, 1936).

Sir Henry Dale (1936) elucidated the mechanisms of normal neuromuscular function, describing the release of acetylcholine at the neuromuscular junction in response to a nerve impulse. An enzyme, cholinesterase, was shown to destroy the neurotransmitter, and Walker (1934) showed that an inhibitor of cholinesterase, physostigmine, was capable of alleviating the muscle weakness characteristic of MG.

That MG may be an autoimmune disease was first suggested by Smithers, (1959), who noted



histological similarities between the myasthenic thymus gland and the thyroid gland of thyroiditis patients. Immunological disorders of MG were described by Nastuk and co-workers (Nastuk et al., 1956, 1960), who reported altered serum complement levels in myasthenic individuals, and by Strauss et al., (1960), who reported the presence of complement-fixing antibody binding to skeletal muscle in vitro. Indirect evidence that MG is an autoimmune disease came from the observation of the increased association of MG with other disorders which were thought to be autoimmune (Simpson, 1960).

Since 1960, our understanding of the physiological defect that causes the symptoms of MG has been greatly improved. The defect at the neuromuscular junction was shown to be localised at the post-synaptic membrane. A simplified morphology of the post-synaptic membrane (Engel and Santa, 1971) and reduced numbers of acetylcholine receptor (AChR) on that membrane (Fambrough et al., 1973; Ito et al, 1978a) were observed. The target of the autoimmune response was confirmed to be the AChR, both by generation of a rabbit model of MG following injection of purified receptor (Patrick and Lindstrom, 1973). Figures 1.3 (a) and (b) illustrate the

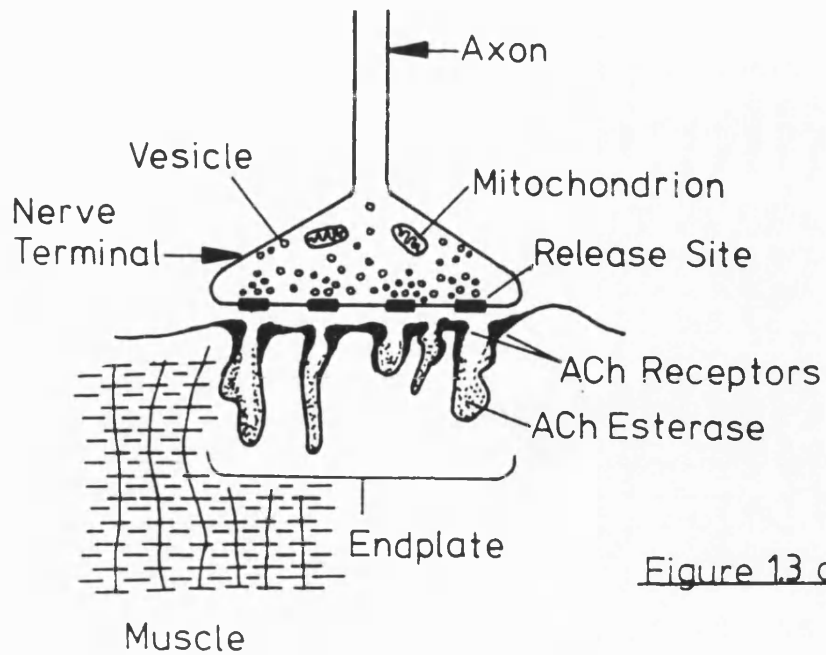


Figure 13 a

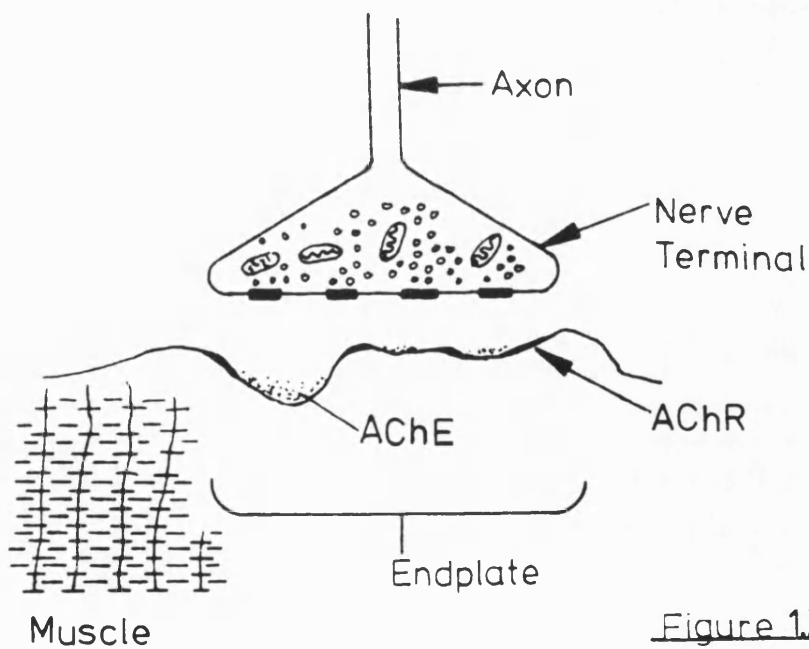


Figure 13 b

Figure 1.3 (a) & (b) A diagrammatic representation of the normal neuromuscular junction (a) and the simplified post-synaptic membrane (b) observed in patients with myasthenia gravis. Note the reduced numbers of acetyl choline receptors (AChR) on the post-synaptic membrane in Figure 1.3(b).

simplification of the post-synaptic membrane and the reduction in the numbers of AChR described above.

### 1.3.3 The Acetylcholine Receptor (AChR)

AChR, isolated from the electric organs of the electric eel, Electrophorus, and the electric ray, Torpedo, has been the subject of extensive characterisation studies which have been comprehensively reviewed (Changeux, 1981; Conti-Tranconi and Raftery, 1982; Barrantes, 1983; Raftery et al., 1983; Anholt et al., 1984). These studies have depended on the properties of certain snake venoms known as  $\alpha$ -toxins, which bind almost irreversibly and with high specificity to the acetylcholine binding site (Lee, 1972). The purity of AChR preparations is usually expressed in terms of the number of  $\alpha$ -toxin binding sites per gram of protein.

The AChR purified from both Torpedo and Electrophorus contains four different subunits,  $\alpha, \beta, \gamma$ , and  $\delta$  with apparent molecular weights of 40,000, 50,000, 60,000 and 65,000. The acetylcholine binding site is located on the  $\alpha$ -subunit (Tzartos and Changeux, 1984; Wilson et

al., 1984 Cahill and Schmidt, 1984). A quaternary structure of  $\alpha_2\beta\gamma\delta$  was proposed by Reynolds and Karlin, (1978) for Torpedo AChR monomers. This structure is represented in its membrane environment in Figure 14. The linkage of two monomers by disulphide bonds between the  $\delta$  subunits is thought to constitute the functional unit in vivo (Schindler et al., 1984). The roles of the  $\beta$ ,  $\gamma$  and  $\delta$  subunits are unknown although one or more of them may be involved in the regulation of the centrally placed cation-channel (Karlin et al., 1983).

Because of the limitations placed on the availability of material, much less is known about the AChR of human muscle. The acetylcholine binding site of mammalian AChR is known to be present on the  $\alpha$ -subunit (Lyddiatt et al., 1979) and early studies on human (Lindstrom et al., 1978) bovine (Lindstrom et al., 1979; Einarson et al., 1982) rat (Einarson et al., 1982) and more recently amphibian (Sargent et al., 1984) AChR preparations have indicated the presence of  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  subunits. The subunits of foetal calf muscle AChR also showed considerable homology with the four subunits of electric fish AChR

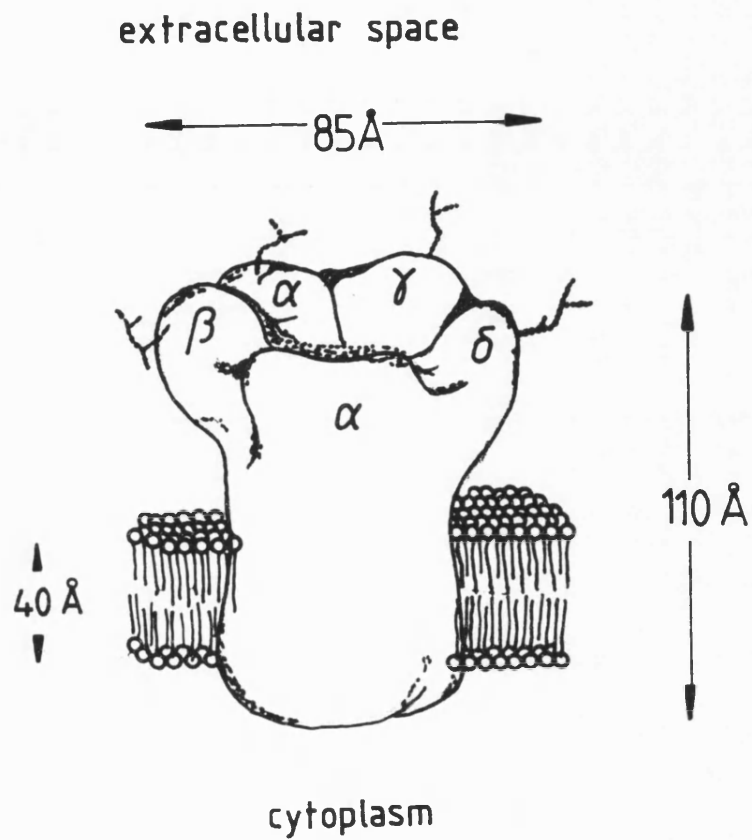


Figure 1.4 . Diagrammatic representation of AChR in the membrane (taken from Harrison and Behan, 1986).

(Conti-Tronconi et al., 1982a). Homology was shown between the  $\alpha$ -subunits of chicken muscle and Torpedo AChR (Barnard et al., 1983).

Interspecies differences were, however, observed (Gullick and Lindstrom, 1982a; James et al., 1983)

More recently, Turnbull et al., (1985) found no major differences between foetal and adult AChR in humans, both of which showed subunits with apparent molecular weights of 44,000, 57,000, 58,000 and 66,000.

#### 1.3.4 Disease characteristics

Approximately 1 in 20,000 of the worldwide population is affected by MG, with no increased frequency in any particular ethnic group. The disease may present over a wide range of ages, although a separate disorder, congenital MG, may occur in males less than 2 years old (Vincent et al., 1981). Myasthenia occurs more frequently in females than males (ratio of 3:2 respectively) and females predominate in cases presenting under the age of 40 years, whilst males predominate in cases presenting over the age of 40. Osserman and Genkins (1971), reported modal values of the

third decade for females and the seventh decade for males. Cases presenting with thymoma have no obvious sex distribution and modal age of onset is in the fifth decade.

#### 1.3.5 Symptoms and disease associations

The characteristic symptom of MG is muscle weakness after repeated use which recovers, although not always completely, with rest. Symptoms are often noticed following an infection or after a period of psychological stress (Campbell and Bramwell, 1900). Penicillamine therapy for rheumatoid arthritis may also induce MG (Bucknall, 1977).

Any striated muscle may be affected; extraocular muscles commonly being the first to become involved. More than half of MG patients present with ptosis or diplopia (double vision), and the majority have eye muscle involvement at some stage of the disease (Simpson, 1960). Difficulty in chewing, swallowing, and breathing occurs, and in severe cases, bowel and bladder sphincter muscles may become involved.

Diagnosis and assignment of disease severity is often performed according to a scale proposed

by Osserman and Genkins (1971) whose degrees of severity range from asymptomatic to I (Ocular signs and symptoms only) to IIA, IIB and IIC. The most severe form IIC, involves severe generalised weakness with a compromise of respiratory function.

The major functional restrictions that are observed are reduced muscular utility apparent upon electrical stimulation of the affected muscles. Abnormally low end-plate potentials (epps) (Elmqvist et al., 1964) were found in myasthenic patients. Although a pre- or post-synaptic lesion could account for the reduced epps and mepps, the observations of Engel and Santa (1971) and Fambrough et al, (1973) favour the post-synaptic lesion theory.

Myasthenia has been reported in association with rheumatoid arthritis (Simpson, 1960), thyroiditis (Aarli et al., 1978), Sjogrens syndrome (Brown et al., 1968) pernicious anaemia (Blecher and Williams, 1967), polymyositis (Behan et al., 1982) and other immune associated disorders. Associations between the development of lymphoid neoplasms in MG patients (Silberstein, 1970) and MG in patients with lymphoma (Davis and Schumacher, 1979) have also been reported.



### 1.3.6 Association with histocompatibility antigens

Disease susceptibility and the presence of certain major histocompatibility complex (MHC) antigens has been linked for over 20 years. Lilly et al, (1964) demonstrated that the susceptibility of mice to Gross virus-induced leukaemia is determined by genes linked to the H-2 locus (analogous to the human HLA locus). Strong associations have since been observed between many autoimmune diseases and the presence of specific HLA determinants (eg HLA-B27 and ankylosing spondylitis, Winchester et al., (1975)).

Myasthenia shows a strong association with HLA-A1 and -B8 (Pirskanen et al., 1972; Fritze et al., 1974; Behan, 1980). A comprehensive review of the detailed HLA associations with MG has been carried out (Harrison and Behan, 1986). The associations that have been observed may differ for various races. The HLA-DR3 antigen has also been associated with MG, although with less frequency than the -B8 antigen (Kaakinen et al., 1975; Dawkins, 1978). However, a recent report of HLA antigens in myasthenic American blacks suggested an association of MG with HLA-A1 and/or

-B8 with an increase in -DR5 rather than -DR3 (Christiansen et al., 1984). In the various races studied, alleles at the -A and -B loci are more prominent than those at the -DR locus.

#### 1.3.7 The role of the thymus in the development of MG

The high incidence of thymitis and thymoma (Castleman, 1966, Namba et al., 1978), in MG and the beneficial effects of thymectomy in many cases of MG (Papatestas et al, 1971a) have indicated the importance of the thymus in the pathogenic process in MG.

Thymic hyperplasia (thymitis) is found in about 80% of all MG cases and is characterised by the presence of germinal centres in the thymic medulla. This condition is generally taken to be diagnostic for MG although small numbers of germinal centres in the thymus have been noted in cases of hyperthyroidism (Vetters and Simpson, 1974) and in a variety of autoimmune diseases (Habu et al., 1971; Tamaoki et al., 1981). Thymoma generally occurs in patients over the age of 40 and the prognosis is poor (approximately 30% of all MG cases) (Mulder et al, 1974).

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### 1.3.8 Humoral responses in MG

MG patients have an increased incidence of autoantibodies directed against targets other than the AChR. This also occurs in other autoimmune diseases and similarly results in accompanying immunological disorders. The incidence of non-AChR directed autoantibodies has been reviewed by Harrison and Behan (1986).

Increased in vivo polyclonal B cell activity has been suggested by Levinson et al., (1981 a,b) with the demonstration of increased levels of spontaneous Ig-secreting cells in 30% of MG patients. However, Harfast et al., (1981) found no spontaneous Ig production in MG patients and Kelly et al., (1981) observed no differences in the spontaneous Ig synthesis by PBL between MG patients and control subjects.

The polyclonal stimulator of B cells, pokeweed mitogen (PWM), is dependent upon T cell processing for its mitogenic properties and this system has been shown to be a useful model for studying regulatory mechanisms of Ig synthesis in vitro (Janossy and Greaves, 1975; Fauci, 1979). Harfast et al., (1981) reported increased PWM-induced Ig production in MG patients compared

to control subjects. Limberg et al., (1985) however, found normal Ig synthesis in MG patients but this was accompanied by decreased IgM synthesis. Kelly et al., (1981) observed no differences in PWM-induced Ig synthesis between thymectomised and non-thymectomised MG patients, both of which were normal.

The spontaneous production of Ig by the peripheral blood lymphocytes (PBL) of MG patients was not observed until day 4 *in vitro*, the kinetics of this resembling that of PWM-induced synthesis (Limburg et al., 1985). This observation was interpreted as a possible T cell involvement in the spontaneous synthesis of Ig.

The ability of unstimulated PBL from patients with generalised MG to synthesise anti-AChR antibodies in vitro has been reported (Lisak et al., 1983; Willcox et al., 1984). However, Newsom-Davies et al., (1981 a,b), reported little or no in vitro anti-AChR antibody synthesis by PBL from MG patients, with or without stimulation by PWM. A thymic cell line producing monoclonal anti-AChR antibody has been established from the thymocytes of an MG patient (Kamo et al., 1982). Whereas only 5-8% of normal subjects synthesise

detectable amounts of anti-AChR antibody after PWM stimulation of PBL (Lisak et al., 1983, 1984), the majority of MG patients PBL synthesise anti-AChR antibody (Lisak et al., 1983) following such stimulation.

Lisak et al., (1984) showed an increase in the anti-AChR antibody secretion by PBL of MG patients after depletion of OKT8<sup>+</sup> cells and following PWM stimulation.

#### 1.3.9 Cellular responses in MG

Selective enhancement by irradiated thymic cells, of anti-AChR antibody production by autologous PBL from myasthenic patients *has been observed* (Newsom-Davis et al, 1981a) and, using monoclonal antibody depletion studies, Willcox et al, (1984) have suggested that this enhancement is mediated by antigen presenting cells rather than by AChR-specific thymic T helper cells. Thomas et al, (1982), proposed that HLA-DR<sup>+</sup> interdigitating cells were involved in antigen presentation although Willcox et al, (1984) argued that germinal centre follicular dendritic cells were primarily responsible for the presentation of the autoantigen. Immuno- histological studies by

Lisak and colleagues showed that some, but not most  $Ia^{+}$  cells in the thymus, express T cell markers. The results presented by Willcox et al, (1984) were interpreted as supporting the hypothesis that the MG thymus might present AChR in a particularly stimulatory fashion and provide a source of potent help to autologous AChR-reactive B cells. A significant increase in the percentage of  $Ia^{+}$  cells in thymic cell suspensions from MG patients has been observed (Lisak et al, 1983) although  $Ia^{+}$  cells were not detected by Bhan et al, (1980).

Abdou et al, (1974), Lisak et al, (1978), Richman et al, (1976) and Opelz et al, (1978) have found that thymic cell suspensions from myasthenic patients stimulate autologous PBL to proliferate in one-way mixed lymphocyte reactions, particularly when these cells are obtained from patients with thymic hyperplasia (Abdou et al, 1974). The stimulus for such an 'autologous MLR' is not known, although the possibilities of a virally induced neoantigen (Lisak and Zwieman, 1976; Lisak et al, 1976) elaboration of a soluble activator or altered thymic AChR have been suggested (Lisak et al, 1976; Newsom-Davis et al, 1981 b), and would be supported by the absence of such an 'AMLR'

between these cells in normal subjects.

Ia<sup>+</sup> cells are potent stimulators of both allogeneic and autologous MLR between PBL (Mingari and Moretta, 1982) and this is of interest when one considers that AChR or altered AChR presented by an Ia<sup>+</sup> dendritic cell could serve as the stimulator.

Surface marker studies, especially those measuring Fc receptors on T cells (T<sub>γ</sub> and T<sub>μ</sub>) (Lisak et al, 1979), T cell markers (OKT4 and OKT8) (Reinherz et al, 1980; Newsom-Davis et al, 1981 a; Lisak et al, 1982; Tindall, 1982; Lisak et al., 1983) and B cell markers (EAC rosettes) (Staber et al., 1975), (IgM/IgD) (Kornstein et al., 1983) have not yielded clear conclusions as to the proportions of these immune cells within the myasthenic thymus. Tables of all the reported changes in T and B cell, suppressor cell and helper cell numbers in MG patients compared to normal controls have been compiled by Harrison and Behan (1986). Conflicting results are apparent, although the majority of reports indicate increased B-cell and decreased T-cell populations, in both the thymus and PBL in MG. Measurements of the helper cell and suppressor cell population sizes by surface marker studies

were found to yield inconclusive results.

Functional assays of suppressor cell activity have indicated impaired suppression by both unstimulated and Con-A stimulated myasthenic lymphocytes on mitogen-stimulated autologous cells (Zilko et al., 1979; Salzner et al., 1981) or on the mixed lymphocyte reaction (Berrih et al., 1981). Defective suppressor cell activity could account for the elevated levels of anti-AChR antibody in MG patients (Miller and Schwartz 1982), but removal of suppressor T cells does not induce anti-AChR antibody synthesis by the PBL of normal individuals (Lisak et al., 1984).

Studies of the responses of myasthenic lymphocytes to non-specific mitogens have been shown to be both normal (Abdou et al., 1974; Lisak and Zweiman, 1975; Kalden et al., 1976; Conti-Tronconi et al., 1979; Trotter et al., 1982) and impaired (Zilko et al., 1979; Berrih et al., 1981; Koethe et al., 1981; Dropcho et al., 1982; Richards et al., 1986). The proliferative responses of lymphocytes from myasthenic patients to AChR has, however, consistently been reported as being significantly greater than that observed in normal controls (Abramsky et al., 1975 a,b; Richman et al., 1976, 1979; Conti-Tronconi et al.,



1977, 1978, 1979, 1982, 1983).

1.3.10 The role of anti-acetylcholine receptor antibody  
in the pathogenesis of MG

Loss of active AChR from the post-synaptic membrane has been suggested to represent the major factor resulting in impaired neuromuscular transmission in MG (Fambrough et al, 1973). A large proportion of the work that has identified the mechanisms involved in this loss was carried out on animal models, and further discussion of the mechanisms may be found in **section 6.5**.

The mechanisms involved in the loss of functional AChR from the post-synaptic membrane may be:

- i) Direct block of AChR
  - ii) Increased degradation or decreased synthesis, or both
  - iii) Complement-mediated lysis of the post-synaptic membrane.
- .....

i) Direct Block

The lack of morphological changes at the muscle end plates and alleviation of symptoms with anticholinesterases suggests that direct block may be contributory in the early stages of the disease process (Nielson et al., 1982.) However, in contrast to the findings in Experimental autoimmune myasthenia gravis (EAMG), few reports have observed the direct block of the electrophysiological response, and those that have may, at least partly, be explained by increased receptor degradation (Bevan et al., 1978).

Reduction in mepp amplitudes by myasthenic sera have been reported with normal human intercostal muscle (Ito et al., 1978 b), myasthenic human intercostal muscle (Sanders et al., 1981), rat diaphragm (Shibuya et al., 1978), and mouse diaphragm (Lerrick et al., 1983). No such reduction in mepp amplitudes by myasthenic sera was observed in rat muscle by Albuquerque et al., (1976).

..... Studies on the binding of cholinergic .....  
ligands to AChR in the presence of myasthenic

sera have utilised the binding of labelled  $\alpha$ -toxin. However, the large size differences between acetylcholine and  $\alpha$ -bungartoxin does not necessarily mean that the behaviour of the two ligands is similar. By using different sources of AChR, Fulpius et al., (1980, 1981), Drachman et al., (1981) and Mittag et al., (1984) have observed some correlation between blocking ability of the serum and the clinical state of the donor. However, the observations of Cull-Candy et al., (1982), of an unchanged myasthenic muscle response to acetylcholine after several days in the absence of anti-AChR antibody, suggests that reversible AChR block does not contribute to reduced neuromuscular function.

ii) Increased degradation of AChR

The increased degradation of muscle AChR in the presence of myasthenic immunoglobulins has been shown to be dependent on temperature, metabolic and cytoskeletal activity but not on complement (Appel et al., 1977; Kao and Drachman, 1977 a). Drachman et al., (1978, 1980, 1982) observed accelerated AChR degradation by

purified myasthenic IgG and by  $F(ab)_2$  fragments but not by Fab fragments. However, cross-linking the Fab-labelled AChR with anti-Fab second antibody resulted in accelerated degradation. The findings of Lowenadler et al., (1981) supported the above observations when, in contrast to myasthenic IgG, the corresponding Fab fragments were found to protect the recipient against EAMG induced by subsequent injection of anti-AChR IgG and did not effect the passive transfer of EAMG to mice. Tzartos et al., (1985) have similarly shown protection of AChR modulation on the mouse muscle cell line, BC3H1, by a Fab fragment directed at the main immunogenic region of the AChR.

Conflicting findings have been reported on the synthesis of AChR. Whereas one report suggested decreased synthesis (Appel et al., 1979), no difference from control synthesis (Drachman et al., 1978 a; Clementi et al., 1982; Sher and Clementi, 1984) and increased synthesis (Fulpuis et al., 1980; Wilson et al., 1983) of AChR by cultured muscle cells in response to myasthenic IgG have been observed.

iii) Complement-mediated membrane lysis

A suggestion of a role for complement in the pathogenesis of MG came with the observation of a fall in complement activity during exacerbation of symptoms in MG patients with normalisation during remission periods (Nastuk et al., 1956, 1960). Evidence of the lytic action of myasthenic serum was noted by Nastuk et al., (1959) with 2 of 22 myasthenic sera showing higher than normal lysis of cultered frog muscle cells. Liveson et al, (1976) reported the lysis of mouse muscle cells by 3 of 17 myasthenic serum samples. An assay developed by Cambridge and Stern (1981) using the specific uptake of radiolabelled carnitine by muscle cells was employed by Childs et al., (1985) to show the lysis of cultured rat myotubes by 9 out of 13 myasthenic sera. These results support the ultrastructural studies of Engel and co-workers (Sahashi et al., 1980) indicating the involvement of complement-mediated membrane lysis in the pathogenic process in MG.

#### 1.4. Aims of the project

The pathogenic effects of anti-acetylcholine receptor antibody in myasthenia gravis are well established, despite the debate over the exact mechanism by which these effects occur. As the other autoimmune diseases, abnormal production of autoantibodies could reflect a breakdown in control exerted by immunoregulatory cells. The AMLR has been suggested as an in vitro correlate of in vivo immune control (Lattime et al., 1982) and, as such, may serve as an important model for examining the defective cellular interactions responsible for a number of autoimmune diseases.

The primary aim of this project was to determine the nature of the defect in cellular immunoregulation in MG, using the AMLR as an indicator. A fundamental requirement for this is the establishment of the normal response in healthy individuals, including the intra- and inter-individual variation in the response. The immune responsiveness of both normal and myasthenic individuals, as assessed by the AMLR, will also be supported by the lympho proliferative responses of the two groups to several mitogens.

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A study of the AMLR in humans with clinically

manifest autoimmune disease precludes the study of disease initiation and development. In an attempt to understand the underlying cellular defects that may occur in the initial stages of the disease, the second aim of this project was to study the AMLR during the induction and subsequent maintenance of EAMG in an animal model. Disease progress can be monitored by both clinical symptoms and the presence of serum anti-acetylcholine receptor antibody. In parallel, the cellular immune responsiveness as assessed by the AMLR and mitogen responsiveness, may indicate the effects of the disease on the immune system.

Whilst the induction of EAMG in an animal by immunisation with the autoantigen, the AChR, may not relate to the aetiology of the disease in humans, the study of the immune responses in EAMG may help in understanding the lack of immunoregulatory control in MG, and possibly other autoimmune diseases.

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2. MATERIALS

$\alpha$ -Bungarotoxin was supplied by the Boehringer Corporation, Lewes, Sussex, U.K.

Naja naja siamensis venom was obtained from the Miami Serpentarium, Miami, Florida, U.S.A.

Carrier free Na<sup>125</sup>I and (methyl-<sup>3</sup>H) thymidine were obtained from the Radiochemical Centre, Amersham, Bucks, U.K.

The following were obtained from the Sigma Chemical Co. Ltd., Poole, Dorset, U.K.:-

2-aminoethylisothiuronium bromide hydrobromide; Papain (EC 3.4.22.2)(Type 111) from Papaya latex, 16-40 units per mg protein; Neuraminidase (EC 3.2.1.18)(Type V) from Clostridium perfringens, 0.5-1.5 units per mg protein; Complete and Incomplete Freund's Adjuvant; Heparin (Grade 1) from Porcine Intestinal Mucosa; L-cysteine hydrochloride.

Sterile filter units (0.2  $\mu$ m) were obtained from Sera-Lab., Crawley Down, W. Sussex, U.K.

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DEAE-cellulose filter discs (DE-81), GF/C



filters and DE52 ion-exchange resin were obtained from Whatman Biochemicals Ltd., Maidstone, Kent, U.K. Benzoquinonium chloride was a gift from Sterling Winthrop, Rensselaer, New York, U.S.A.

The following were obtained from Flow Laboratories, Irvine, Scotland:-

RPMI 1640 (Dutch Modification); Penicillin /Streptomycin; Human AB serum; multi-well cell harvester; glass fibre cell harvester filter mats; sterile screw-top centrifuge tubes (10ml); sterile plastic Pasteur pipettes; lymphocyte separation medium (Ficoll-Hypaque).

Foetal Calf Serum and NUNC 96-well microtitre plates were obtained from Gibco Ltd, Paisley, Scotland.

NU-SERUM was obtained from Collaborative Research Inc., Lexington, MA, U.S.A.

Phytohaemagglutinin-P and LPS (Type W from S. typhimurium) were obtained from Difco Ltd., Detroit, U.S.A.

Sephadex-G25 and Concanavalin-A were obtained from Pharmacia Ltd., Milton Keynes,

Bucks, UK.

Rabbit anti-human IgG (F(ab)<sub>2</sub> fragment) fluorescein conjugate was obtained from Behringwerke AG, Marburg, W. Germany.

Mouse anti- (rabbit T cell) monoclonal antibody (IgG2a), as ascitic fluid, was obtained from RIA (U.K.) Ltd., Tyne & Wear, U.K.

Sheep red blood cells and ox red blood cells were obtained from Tissue Culture Services, Slough, Berks, U.K.

Folin and Ciocalteus' phenol reagent was obtained from BDH Chemicals Ltd, Poole, Dorset, U.K.

LKB Optiphase 'Safe' Scintillation fluid was obtained from Fisons plc, Scientific Equipment Division, Loughborough, U.K

Nylon wool (Leuko-Pak) was obtained from Travenol Laboratories.

2.1. Buffer CompositionsTris ammonium chloride erythrocyte lysis buffer

Tris (hydroxymethyl) aminomethane 0.17M  
(20.6 g/L) ammonium chloride 0.16M (8.3 g/L).

Add 0.17M Tris buffer (10ml) to 0.16M  
ammonium chloride (90ml) and adjust pH to 7.2.  
Sterilise by autoclaving to 20lbs/sq inch for 20  
min.

Phosphate buffered saline (PBS), pH 7.2, 0.15M

NaCl	8 g/L
KCl	0.2g/L
Na <sub>2</sub> HPO <sub>4</sub>	1.15g/L
KH <sub>2</sub> PO <sub>4</sub>	0.2g/L

Make up with double distilled water and  
adjust to pH 7.2. Sterilise by autoclaving at  
20lbs/sq inch for 20 min.

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Balanced Salt Solution (BSS)

Glucose	1g/L
CaCl <sub>2</sub>	0.14g/L
NaCl	8g/L
KCl	0.2g/L
MgSO <sub>4</sub> 7H <sub>2</sub> O	0.2g/L
MgCl <sub>2</sub> 6H <sub>2</sub> O	0.2g/L
KH <sub>2</sub> PO <sub>4</sub>	0.06g/L
Na <sub>2</sub> HPO <sub>4</sub>	0.24g/L

Membrane filter (0.22µm) to sterilise

### 3. METHODS

#### 3.1. Aseptic manipulation of lymphocytes and media

All aseptic techniques were performed in an InterMed Microflow Pathfinder class II laminar air flow cabinet.

#### 3.2. Separation of peripheral blood mononuclear cells

Mononuclear cells were isolated by the method of Boyum (1968). Peripheral venous blood (20ml) was taken and mixed with heparin (20U/ml). Heparinised blood (4ml) was diluted with balanced salt solution (4ml), layered onto lymphocyte separation medium (3ml) (Ficoll-Hypaque) in sterile, 15ml polycarbonate screw-top centrifuge tubes and centrifuged at 400g for 30 min at room temperature. The mononuclear cells were harvested from the interface, washed three times in RPMI 1640 and then resuspended at  $3 \times 10^6$  cells/ml in medium consisting of RPMI 1640, 1mM L-glutamine, 100 U/ml Penicillin, and 100  $\mu$ g/ml Streptomycin, (complete medium).

### 3.3. Separation of human T and non-T leucocytes by rosette formation

#### 3.3.1 Papain treatment of sheep's red blood cells (SRBC)

SRBC were treated with papain according to the method of Wilson et al (1975). Packed, washed SRBC from whole sheep's blood (approximately 10ml) were resuspended in RPMI 1640 to their original volume. A sample of the SRBC suspension was removed and resuspended to 10% (v/v in PBS) in RPMI 1640. A sterile solution of papain (1.0% v/v in PBS) was mixed thoroughly with a 2% (w/v) solution of cysteine hydrochloride in PBS and then mixed with an equal volume of the 10% suspension of SRBC. The mixture was incubated for 12 min at 37°C and the cells were then washed twice in RPMI 1640 and resuspended to 2.5% (v/v) in RPMI 1640. The cells were suitable for use for up to 5 days after papain treatment.

#### 3.3.2 AET-Treatment of SRBC

SRBC were treated with AET according to a modified method of Kaplan and Clark (1974). 140mM 2-aminoethylisothiuronium bromide hydrobromide (AET) was prepared by dissolving crystalline AET (0.5g) in double-distilled water (12.5ml). The

pH of this solution was adjusted to pH 9.0 with 10M NaOH. Packed, washed SRBC from whole sheep's blood (approximately 10ml) in Alsever's solution were resuspended in freshly prepared, filter-sterilised, AET solution (10ml). During washing of the SRBC, care was taken to ensure the complete removal of the buffy coat cells. The cell suspension was then placed in a 37°C water bath for 15 min, following which the treated SRBC were washed in PBS four times, or until any residual haemolysis ceased. A stock suspension of the SRBC-AET was produced by resuspending the packed cells to their original volume in complete medium containing 10% FCS (v/v). A 2% (v/v) suspension of SRBC-AET in complete medium containing 40% FCS (v/v) was produced from the stock suspension and this was used as the rosetting medium. When stored at 4°C, untreated SRBC suspensions were suitable for use for up to 2 weeks and AET-treated SRBC suspensions were suitable for use for 1 week.

### 3.3.3 Neuraminidase-treated SRBC

The preparation of neuraminidase-treated SRBC was carried out according to a modified method of Galilu and Schlesinger (1974). A suspension of SRBC in Alsever's solution was washed three times

in RPMI 1640 care being taken after each wash to remove the buffy coat layer. The packed, washed SRBC were then resuspended to their original volume in RPMI 1640 and an aliquot was removed and resuspended to 10% (v/v) in RPMI 1640. Neuraminidase (EC.3.2.1.18) type V was then added to the 10% (v/v) SRBC suspension at a concentration of 10 U/ml. The solution was mixed and incubated for 45 min at 37°C. After incubation, the cells were washed twice with RPMI 1640 and then resuspended to 2.5% (v/v) in RPMI 1640. Neuraminidase-treated SRBC were suitable for use for up to two weeks when stored at 4°C.

#### 3.4. Separation of human T-lymphocytes and non-T leucocytes using native or treated SRBC

The isolated human PBL were separated by rosetting with SRBC. SRBC suspension (2ml) and the suspension of human PBL ( $3 \times 10^6$  cells/ml; 2ml) were mixed thoroughly in sterile 15ml polycarbonate screw-top centrifuge tubes and left overnight at 4°C. The pellet was then carefully resuspended in the supernatant by using a sterile glass Pasteur pipette. The resuspended cells (4ml) were carefully layered onto Lymphocyte Separation Medium (3ml) in sterile, polycarbonate screw-top centrifuge tubes and centrifuged at



400g for 30 min at room temperature. The unrosetted non-T cells were removed from the interface by carefully aspirating off the grey band by using a sterile, glass Pasteur pipette. The supernatant was removed from the remaining cell pellet and the cells were resuspended in RPMI 1640 before transferring to a sterile 30ml plastic universal container. These cells (putative T cells) and the non-T cells (interface cells) were washed once with RPMI 1640, resuspended in Tris-buffered ammonium chloride (0.83%) lysis buffer, and left for 10 min at room temperature. Both cell populations were then washed twice in RPMI 1640. The cell concentration was, in each case, determined by using a counting chamber and the viability of each population was determined by trypan blue dye exclusion. Both cell populations were then resuspended to the desired cell concentrations in the appropriate culture medium. Non-T cells and a proportion of the T cells were inactivated in a calibrated  $^{60}\text{Co}$  gamma ray source (see Methods p 69 ).

All cell suspensions were plated out, by using alcohol-cleaned Hamilton repeater syringes, into sterile, plastic, 96-well round bottom

microtitre plates with lids. Each well contained a total of 200 $\mu$ l of culture medium. The cultures were incubated at 37°C in a humidified atmosphere of 2.5% CO<sub>2</sub> in air for the whole of the culture period.

### 3.5. Separation of T and non-T cells by nylon wool passage

The method of Julius et al (1973), utilising nylon wool to separate T and non-T cells was investigated as an alternative method to rosetting with SRBC. Human PBL from heparinised venous blood were isolated as previously described (Methods, p 57 ). Nylon wool was vigorously washed alternately in distilled water and 0.9M HCl over a period of 2-3 days. The washed wool was then boiled in several changes of distilled water, dried in an oven and pieces (0.6g) were packed into the barrels of 20ml syringes up to the 6ml mark. These nylon wool columns were then separately packaged and autoclaved. When required, each column was washed through with complete medium (25ml). Medium (approximately 2ml) was allowed to remain on the top of the nylon wool and the column was sealed at both ends. The sealed column was incubated at 37°C for 1hr, and then flushed with

fresh complete medium (5ml).

The isolated PBL ( $20-30 \times 10^6$ ) were resuspended at  $1-5 \times 10^6$  cells/ml in complete medium, placed on the column and allowed to drain into the nylon wool. The column was resealed and incubated at  $37^\circ\text{C}$  for 45 min, following which the cells were eluted from the column with complete medium (20ml). A sterile hypodermic needle (23G) was attached to the end of the syringe to control the rate of flow of the eluate. The eluted cells were washed in RPMI 1640 and resuspended at  $1 \times 10^6$  cells/ml in complete medium containing 10% (v/v) NU-SERUM. The nylon wool non-adherent eluate cells were classed as T cells. The nylon wool was removed from the syringe barrel and teased in warm complete medium (10ml) on a sterile Petri dish (9cm). The cells obtained from the nylon wool were washed twice in RPMI 1640 and resuspended at a concentration of  $1 \times 10^6$  cells/ml in complete medium containing 10% (v/v) NU-SERUM.

### 3.6. Determination of surface markers

The surface markers present on separated and unseparated PBL populations were determined in order to characterise the cells present. The E-rosette test, surface immunoglobulin staining,

and endogenous peroxidase staining were employed to detect T cells, B cells and monocytes respectively.

The E-rosette test was performed in duplicate according to the method of Madsen and Johnson, (1979). A 2% (v/v) suspension of AET-treated SRBC in complete medium containing 40% (v/v) FCS (100 $\mu$ l) was mixed with the leucocyte populations at  $3 \times 10^6$  cells/ml in complete medium (100 $\mu$ l) in LP3 tubes. The tubes were then centrifuged at 50g for 5 min and incubated overnight at 4°C. Immediately prior to counting, one drop of toluidine blue (0.2% w/v in 0.9% w/v saline) was added to each tube and the pellet was resuspended by gentle manual shaking. The cell suspension was then counted as described (Methods, p66). At least 200 leucocytes were counted and those cells with three or more SRBC tightly bound were considered to be rosette forming cells.

Surface immunoglobulin-positive staining cells and endogenous peroxidase-positive staining cells were detected according to the method of Waller and MacLennan, (1977). Isolated PBL were washed three times in warm PBS and then incubated for 1hr in RPMI 1640. A dilution (1/4) of

fluorescein-conjugated rabbit anti-human IgG antibody (Fab<sub>2</sub>) (100μl) in PBS containing 0.02% (w/v) sodium azide was added to 2x10<sup>6</sup> pelleted leucocytes, mixed thoroughly and incubated for 20-30 min on ice. The cells were centrifuged three times for 10 min at 200g in cold PBS, containing 0.02% (w/v) sodium azide, and the cell pellet was resuspended in one drop of heat-inactivated FCS. The cell suspension was smeared onto a clean, dry, glass microscope slide and air dried. Surface immunoglobulin positive staining cells were counted as those cells showing cell surface immunofluorescence by using a Zeiss fluorescent microscope with epi-illumination. The cells were fixed in methanol for 10 sec, and rinsed in PBS. The fixed cells were then stained for endogenous peroxidase. Fresh stain solution was prepared by dissolving 3,3' diaminobenzidine (6mg) in Tris-buffer (10ml), and 2 drops of 10 vol. hydrogen peroxide were added immediately before use. The smears were flooded with the fresh stain for 10 min at room temperature and then rinsed in 10% (w/v) saline. The smear was mounted in one drop of buffered glycerol (12 parts glycerol to 9 parts PBS) and a coverslip sealed above the smear by using nail varnish. Positive staining endogenous peroxidase cells appeared

brown under the light microscope (x40 magnification).

### 3.7. Cell counting and cell viability

An appropriate dilution of the cell suspension ( $10^6$  -  $10^7$  cells/ml) was counted by mixing the cell suspension (50 $\mu$ l) with 0.01% (w/v) methylene blue (50 $\mu$ l) in 1% (v/v) acetic acid. At least 200 lymphocytes were counted by using a rhodium-plated improved Neubauer haemocytometer. Cell viability was determined by incubating the diluted cell suspension (50 $\mu$ l) with 0.2% (w/v) trypan blue in PBS at 37°C for 5 min. Viable cells exclude the dye, dead cells appear grey-blue and have a ragged appearance.

### 3.8. Autologous mixed lymphocyte cultures

The AMLR was measured in both normal and myasthenic PBL by using a modified method of Opelz et al, (1975).

T cells ( $1 \times 10^5$ ) were cultured with  $1 \times 10^5$  irradiated (2500 rad) non-T cells in complete medium (200 $\mu$ l) containing 10% (v/v) NU-SERUM. Quadruplicate cultures were carried out in round bottom microtitre plates in 2.5% (v/v) CO<sub>2</sub> in air

at 37°C, over a period of nine days, each 24h culture period being established on a separate microtitre plate. T cells ( $1 \times 10^5$ ) cultured with irradiated T cells ( $1 \times 10^5$ ) in complete medium (200 $\mu$ l) containing 10% (v/v) NU-SERUM were used for T cell controls. Non-T cells ( $2 \times 10^5$ ) in complete medium (200 $\mu$ l) containing 10% (v/v) NU-SERUM were used for non-T cell controls. Culture medium replenishment was carried out on days 4 and 7 of the time course response. On each of these days, culture medium (100 $\mu$ l) was removed from each well and replaced with fresh, warm culture medium (100 $\mu$ l). The cultures were pulsed with [ $^3$ H]-thymidine and harvested 24h later as described (Methods, p68).

### 3.9. Determination of optimum mitogen concentrations

The optimal concentrations of phytohaemagglutinin-P, concanavalin-A, and lipopolysaccharide for stimulation of purified T and non-T cells were determined. Cells ( $2 \times 10^5$ ) were cultured in complete medium (200 $\mu$ l) containing 10% (v/v) NU-SERUM over a period of four days. A range of concentrations was tested for PHA-P (0.1-1.0%), Con-A (2.5-4.0 $\mu$ g/ml) and LPS (5-25 $\mu$ g/ml) for both T cells alone and non-T cells alone. Controls consisted of T cells

( $1 \times 10^5$ ) and non-T cells ( $1 \times 10^5$ ) cultured in complete medium (200 $\mu$ l) containing 10% (v/v) NU-SERUM in the absence of added mitogen. Twenty four hours prior to harvesting, the mitogen-stimulated and unstimulated cells were pulsed with radiolabel (Methods, p68). The cells were harvested as described (Methods, p68 ).

### 3.10. Labelling and harvesting of the pulsed cultures

Twenty four hours prior to the termination of each culture, [ $^3$ H]-thymidine (1 $\mu$ Ci, 5 Ci/mmol) in complete medium (20 $\mu$ l) containing 10% (v/v) NU-SERUM was added to each well and the cultures were subsequently harvested onto glass-fibre filter paper, by using a multi-well semi-automated cell harvester. The filter mat was left to dry and each glass fibre disc, corresponding to each well harvested, was punched out of the filter mat and placed in a polyethylene scintillation vial. Scintillation fluid was added (5ml) and the vial shaken. Tritiated thymidine uptake was measured in a liquid scintillation counter (Packard TriCarb, Model 3255). The counting efficiency for [ $^3$ H] was 30%.



### 3.11. Calculation of the net AMLR ( $\Delta$ cpm) and net mitogen-induced responses

The  $\Delta$  cpm AMLR responses were calculated by subtracting the mean of the duplicate T cell control responses from the mean of quadruplicate AMLR cultures (T cells with irradiated non-T cells).

Net mitogen induced responses were calculated by subtracting the means of duplicate T cell or non-T cell controls from the means of duplicate mitogen-stimulated T cells or mitogen stimulated non-T cells, respectively.

### 3.12. Calibration of a $^{60}\text{Co}$ gamma ray source

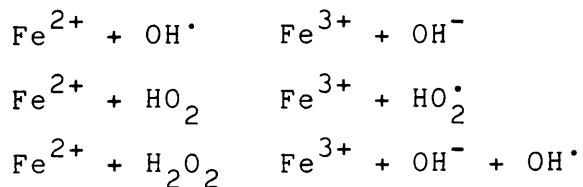
The  $^{60}\text{Co}$  gamma ray source used for irradiation of the cells was calibrated by using the ferrous sulphate dosimetry method of Arena (1971). Stock solutions of 10mM NaCl and 4M  $\text{H}_2\text{SO}_4$  were prepared in double-distilled water. A fresh dosimeter was prepared immediately prior to use by dissolving ferrous ammonium sulphate (0.985g) in stock NaCl (25ml) and  $\text{H}_2\text{SO}_4$  (25ml). The solution was made up to 250ml with double distilled water and thoroughly mixed. This Fricke dosimeter (10ml) was placed in a plastic

universal tube similar to those which would be used for future irradiation procedures for live cells. The tube was irradiated for 10 min. At 2 minute intervals, the tube was removed from the irradiation chamber and samples (1ml) of the solution were removed. The absorption of each sample at 305 nm was then measured by using a CECIL CE272 UV spectrophotometer. The assay blank consisted of non-irradiated dosimeter solution.

### 3.13. Calculation of absorbed radiation dose

The absorption of ferric ( $\text{Fe}^{3+}$ ) ions is strong in the ultra-violet region, whereas that of  $\text{Fe}^{2+}$  ions is negligible.

The ionization of ferrous ions by irradiation with  $\gamma$ -rays relates to the formation of free radicals in solution as below:



The delivered dose of radiation is given by the equation:

$$\text{Dose (rads)} = \frac{A \cdot k}{E \cdot p \cdot G}$$

where:

A = Absorbance of the irradiated solution

k = the product of Avogadro's number and the number of ergs/electron volt =  $9.65 \times 10^8$

E = Molar extinction coefficient of  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  solution at 305mm,  $25^\circ\text{C}$   
= 2196 (+0.6%/°C).

p = Density of working solution = 1.024.

G = G value for Cobalt-60 = 15.5 at  $15-25^\circ\text{C}$ .  
(ions formed per 100eV absorbed.)

#### 4. RESULTS

##### 4.1. Separation of human T-lymphocytes and non-T leucocytes: E-rosette separation

The effects of various reagents on the ability of washed sheep red blood cells (SRBC) to rosette with human peripheral blood lymphocytes (PBL) were investigated. Unsensitised SRBC have been shown to bind specifically to human T lymphocytes, although wide variation has been reported in the percentage of rosette forming cells (RFC) found in human PBL isolated from normal donors by different investigators (Jondal et al., 1972; Papamichail et al., 1972).

Papain-treated (Wilson et al., 1975), neuraminidase-treated (Galilu and Schlesinger, 1974), AET-treated (Kaplan and Clark, 1974), and native SRBC (Brain et al., 1970), were used to determine the most effective treatment of SRBC for optimal rosetting with human T lymphocytes. Each treatment was performed on erythrocytes from the same SRBC sample so as to avoid variations in SRBC quality. All treated and native SRBC were rosetted overnight at 4°C with PBL from three healthy donors. The results, expressed as the percentages of E-RFC, surface membrane

immunoglobulin positive, endogenous peroxidase-staining and null cells present in both the putative T and non-T cell populations are shown in Table 4.1.

As seen in Table 4.1, the greatest purity of the T cell population, according to surface marker studies was produced by treatment of the SRBC with neuraminidase, although treatment of the SRBC with AET yielded T cell populations of a very similar purity.

The T cell population obtained by using neuraminidase- and AET-treated SRBC resulted in rosettes which were very stable to manual manipulation and which appeared as dense 'morula'-like rosettes with more than ten red cells bound per lymphocyte. The individual variation between the three human PBL samples studied was very small for both AET- and neuraminidase-treated SRBC, whereas the variation observed with both papain-treated and native SRBC was considerable. Contamination by the other cell types in the rosetted and non-rosetted populations was also significant for both the papain treated and the native SRBC. These two alternative rosetting methods were accordingly considered unsuitable for use as a routine

TABLE 4.1

The composition of separated T and non-T cell populations following separation by various forms of rosetting with SRBC treated as shown.

TREATMENT	<u>T cell (%)</u>			<u>Non-T cell (%)</u>		
	<u>E-RFC</u>	<u>SmIg+ve</u>	<u>EP+N</u>	<u>E-RFC</u>	<u>SmIg+ve</u>	<u>EP+N</u>
PAPAIN	88 $\pm$ 7.6	6 $\pm$ 3	1	8.4 $\pm$ 0.5	76 $\pm$ 2.1	13.5 $\pm$ 1
NEURAMINIDASE	98 $\pm$ 2.1	1.5 $\pm$ 0.9	0	2.9 $\pm$ 0.2	87 $\pm$ 1	14 $\pm$ 0.4
AET	96 $\pm$ 3.8	2.2 $\pm$ 0.2	0	1.6 $\pm$ 1.3	87.5 $\pm$ 1.3	12 $\pm$ 0.9
NATIVE	82.3 $\pm$ 6.8	7.4 $\pm$ 1.6	2	10 $\pm$ 2.7	74 $\pm$ 8.3	8 $\pm$ 1.3

Legend: Values represent means  $\pm$  s.e.m. on 7 observations for each treatment.

Abbreviations used: E-RFC - erythrocyte rosette forming cells; SmIg+ve - surface membrane immunoglobulin positive cells; EP+N - endogenous peroxidase positive plus null cells.

separation procedure.

The instability of the rosettes produced with papain-treated SRBC and native SRBC probably contributed to the observed contamination of the T and non-T cell populations, because of the disruption of rosettes during the resuspension of the rosetted pellet. Microscopic examination of the rosettes in these cases showed no more than four red cells bound per lymphocyte, most rosettes having 2 or 3 red cells bound.

As this rosetting procedure was intended to be developed into a routine assay for separating human T and non-T PBL, the cost of performing the assay must not be prohibitive. However, the use of neuraminidase in the quantities required resulted in its use becoming highly uneconomical. This, together with the similar performance of AET-treated SRBC with human peripheral blood leucocytes favoured the use of AET as the 'activating' substance for treatment of the SRBC. Thus, all routine rosetting assays were carried out using SRBC AET as the separating red cells.

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#### 4.2. Development of conditions for the treatment of SRBC with AET

Various concentrations of AET over a pH range of 7-11 were tested with SRBC to determine the optimum conditions necessary for AET treatment of SRBC.

As shown in Table 4.2, high concentrations of AET resulted in gross haemolysis of the SRBC. This may have arisen from the direct action of the sulphhydryl compound on the membrane of the red cells or as a result of extreme 'stickiness' of the red cells. This 'stickiness' resulted in difficult resuspension of the red cell pellet during the washing procedures, and the excessive manual manipulation required to resuspend the red cell pellet may have ruptured the red cell membrane. Within the range of values tested, pH did not have a dramatic effect on the haemolytic effect of AET but the most stable rosetting occurred at pH 9.0, (Table 4.2.). Thus, the optimum conditions used for treating SRBC for use in a routine assay with human PBL were 140mM AET at pH 9.0.

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AET Concentration (mM)

<u>pH</u>	35	70	140	210	280
7	+/*	+/**	+/***	+++/*	++++/*
8	+/*	+/***	+/***	+++/**	++++/*
9	+/**	+/***	+/****	+++/**	++++/*
10	+/**	+/***	++/***	+++/**	++++/*
11	+/**	+/***	++/***	+++/**	++++/*

Table 4.2 Chessboard titration of AET concentration (mM) and pH of the AET solution used for SRBC treatment. Each combination of results indicates the degree of haemolysis as the plus symbol (+ to +++, slight to severe) and efficiency of rosette formation as the asterisk symbol (\* to \*\*\*, poor to excellent). Efficiency of rosette formation was determined by the stability of rosettes formed and the number of SRBC-AET bound per lymphocyte.

The centrifugation conditions used in the washing procedures following treatment of the SRBC were found to affect the degree of haemolysis. Centrifugation at 100g for 15 min for the first wash, followed by washes at 200g for 10 min were found to produce the least haemolysis (data not shown).

Thus, all subsequent rosetting procedures for the separation of human T and non-T cells were performed using the optimum conditions for SRBC treatment.

#### 4.3. Determination of the optimum mitogen concentration for stimulation of T -cells and non-T cells by PHA-P, Con-A and LPS

The proliferative responses of SRBC<sub>AET</sub> - separated human peripheral blood T lymphocytes and non-T leucocytes to a range of concentrations of the mitogens PHA-P, Con-A and LPS were investigated to determine the optimum concentration of each mitogen for routine use.

Fig 4.1 (a) shows the mean responses of non-T cells, from three individuals, to a range of concentrations of PHA-P. T-cell proliferative responses were observed between 0.1% and 0.8%

**Figure 4.1(a)**

Proliferative responses of separated T ( $2 \times 10^5$ ) cells and non-T ( $2 \times 10^5$ ) cells, to the mitogen phytohaemagglutinin-P (PHA-P).

**Figure 4.1(b)**

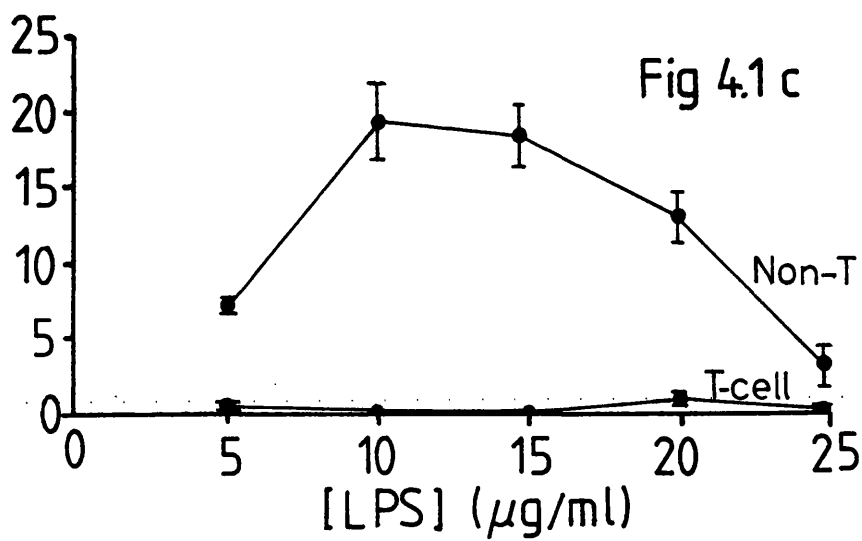
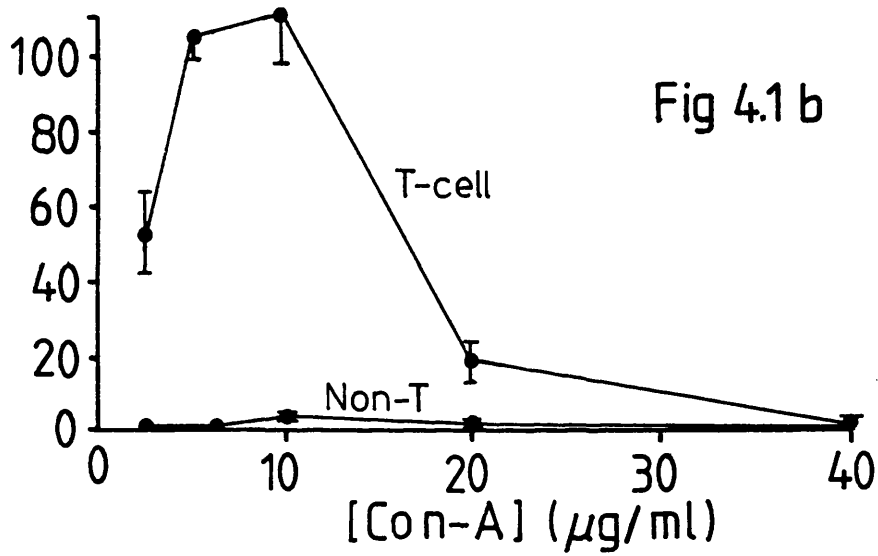
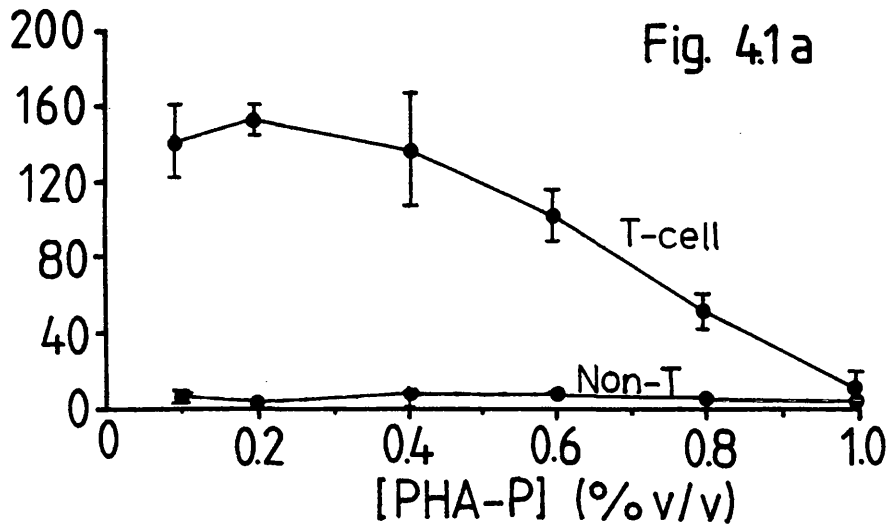
Proliferative responses of separated T ( $2 \times 10^5$ ) cells and non-T ( $2 \times 10^5$ ) cells, to the mitogen concanavalin-A (Con-A).

**Figure 4.1(c)**

Proliferative responses of separated T ( $2 \times 10^5$ ) cells and non-T ( $2 \times 10^5$ ) cells, to the mitogen lipopolysaccharide (LPS).

The ordinate value represents the mean of the sum of duplicate tests of cells ( $2 \times 10^5$ ) with mitogen minus the mean of the sum of duplicate tests of cells ( $2 \times 10^5$ ) in the absence of mitogen. Values are means of results obtained with cells from three individuals; the vertical bars represent the s.e.m. for the three individuals' responses.

[<sup>3</sup>H]-Tdr  
Uptake ( $\times 10^{-3}$ )



PHA-P (v/v) with a peak response at 0.2% (v/v). Non-T cell responses were much lower at all mitogen concentrations, the highest response being at 0.4% (v/v) PHA-P. This response could arise from the proliferation of B cells, induced by minor contamination ( $<3\%$ ) of activated T cells or by direct stimulation of B cells with PHA-P.

T cells incubated with Con-A showed a peak response with  $10\mu\text{g/ml}$  mitogen (Fig 4.1(b)). Response was similar at  $5\mu\text{g/ml}$  but much lower at higher concentrations ( $20\mu\text{g/ml}$  and  $40\mu\text{g/ml}$ ) of Con-A. The non-T cell response with Con-A was very low at all concentrations.

The response of T cells and non-T cells to LPS are shown in Fig 4.1 (c). T cells incubated with LPS showed no response at all the concentrations of the mitogen. However, a peak response at  $10\mu\text{g/ml}$  was observed for non-T cells.

The data shown in Fig 4.1. indicate that the rosette separated leucoctye populations were purified both with regard to surface markers and mitogen responsiveness.

#### 4.4. Separation of cell populations by nylon wool

The T-lymphocyte and non-T leucocyte populations were separated by using the nylon wool method described by Julius et al (1973). Table 4.3 shows the result of surface marker studies and Figure 4.2 represents the optimal mitogenic responses of the nylon wool separated cell populations.

As seen in both Table 4.3 and Fig 4.2, the separation afforded by the nylon wool method was worse than that achieved by rosetting. This method was accordingly considered to be unsuitable for routine use and rosetting by AET-treated SRBC was subsequently routinely used for separation of human T lymphocytes and non-T leucocytes.

#### 4.5. Irradiation of T and non-T cells

The  $^{60}\text{Co}$  gamma ray source was calibrated according to the method of Arena (1971) (Methods, p 69 ). Figure 4.3 shows the calibration curve for duplicate samples of the dosimeter irradiated for 10 min with 2 min sample intervals. The dose rate (rads/minute) was determined from the slope by using the equation described (Methods,

	T cell (NUNA)	Non-T cell (NA)
SmIg+	24%	66%
E-RFC	60%	15%
E. Perox.	8%	12%
Null	8%	7%

Table 4.3 The percentages of the various cell types as measured by surface markers and endogenous peroxidase activity in nylon wool non-adherent (NUNA) and nylon wool adherent (NA) cell populations.

Abbreviations used:

SmIg+ : Surface membrane immunoglobulin  
positive cells

E-RFC : Erythrocyte rosette forming cell

E.Perox. : Endogenous Peroxide-containing cells

Null : Null cells.

Figure 4.2. Mitogenic responses of nylon wool non-adherent (NNA) ( $2 \times 10^5$ ) and nylon wool adherent (NWA) ( $2 \times 10^5$ ) cells to optimum concentrations of the mitogens PHA-P, Con-A and LPS. Optimum concentrations of mitogens were as previously determined.

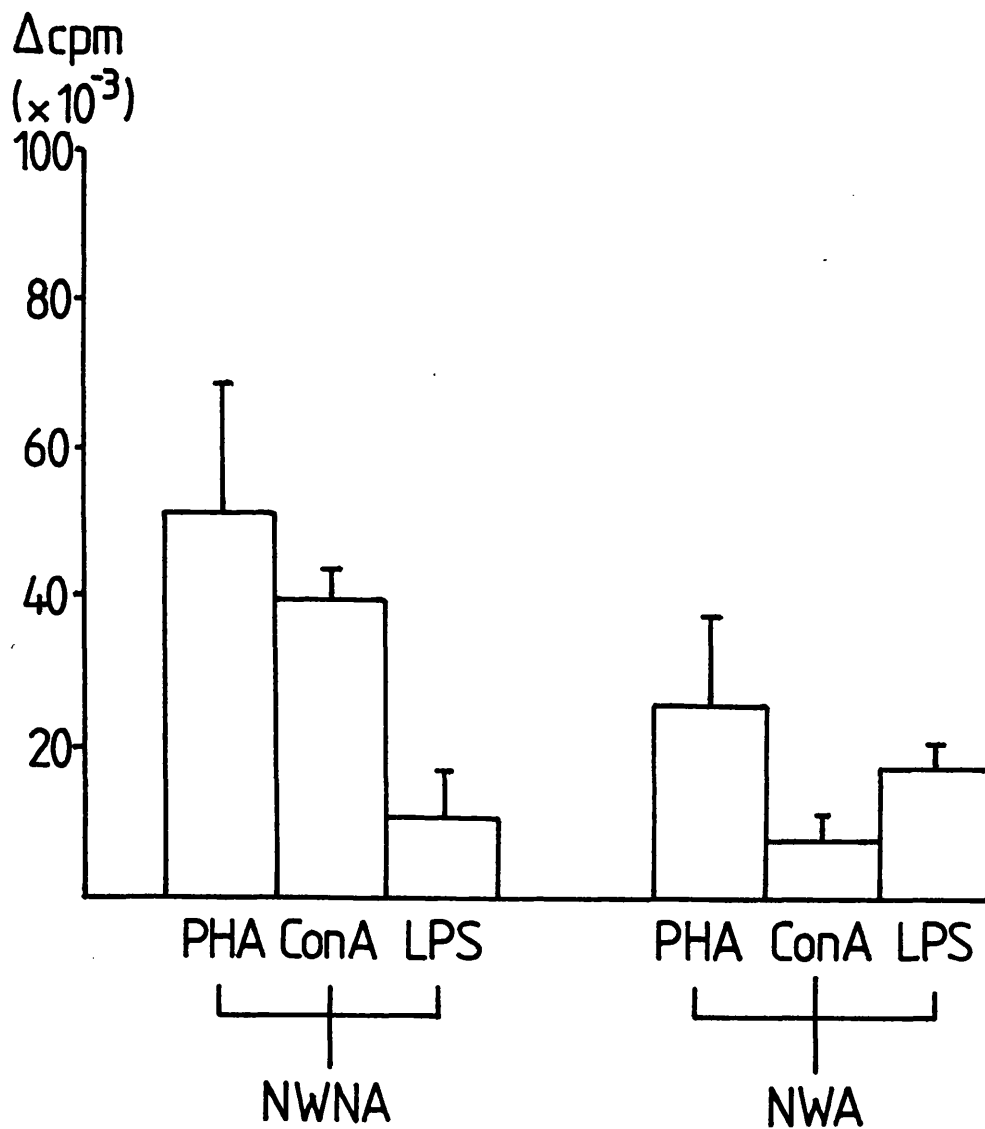
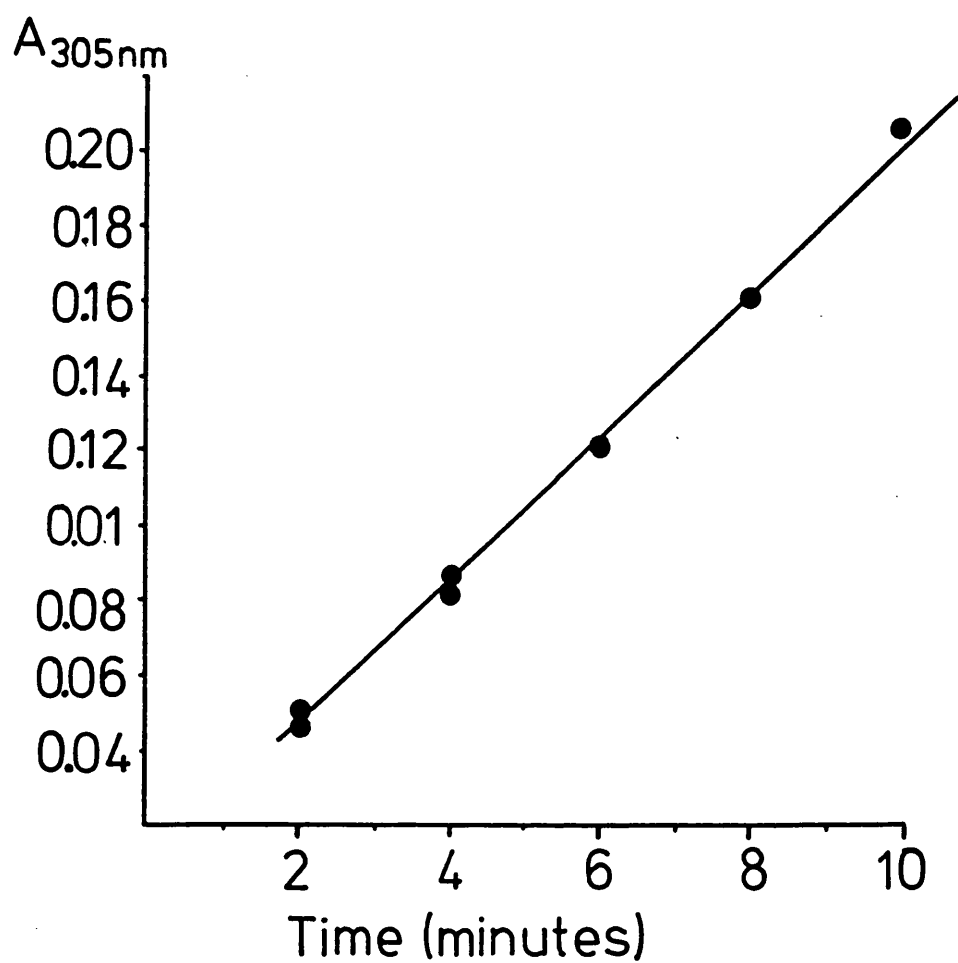




Figure 4.3. The calibration curve for the Fricke Dosimeter. All the irradiations were carried out in a plastic 30ml universal tube similar to those used for irradiation of cell samples.



p71).

#### 4.6. Development of standard conditions for use in a routine AMLR assay

A standard set of conditions was developed in order to enable the direct comparison between AMLR responses obtained for normal healthy individuals and myasthenic patients. The factors which were investigated were:

- 1) Type and concentration of serum supplement in the culture medium,
- 2) Numbers and ratios of T and non-T lymphocytes in each culture.

##### 4.6.1 Effect of serum supplement type and concentration

The responses observed for various types of serum supplement were studied over an eight day period (2-9 days in culture) and are shown in Figs 4.4 and 4.5.

Figure 4.4(a) represents the mean AMLR response observed when the cells were cultured in complete medium containing 10% (v/v) NU-SERUM. The AMLR was low for the first 4 days but

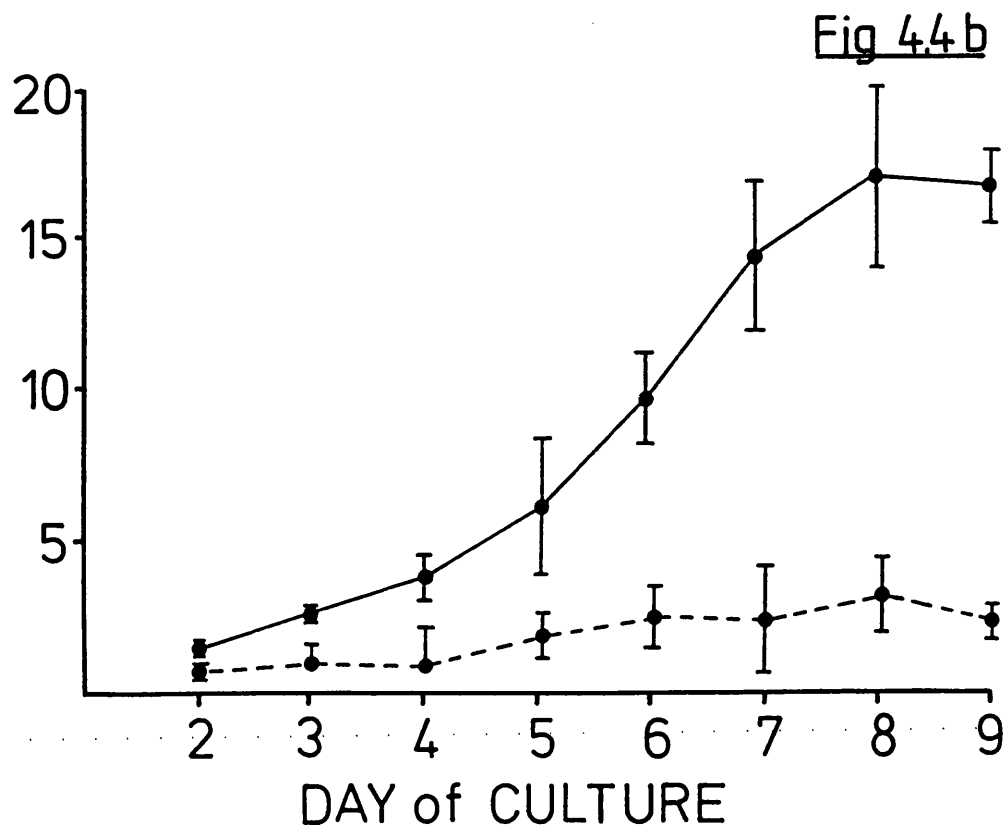
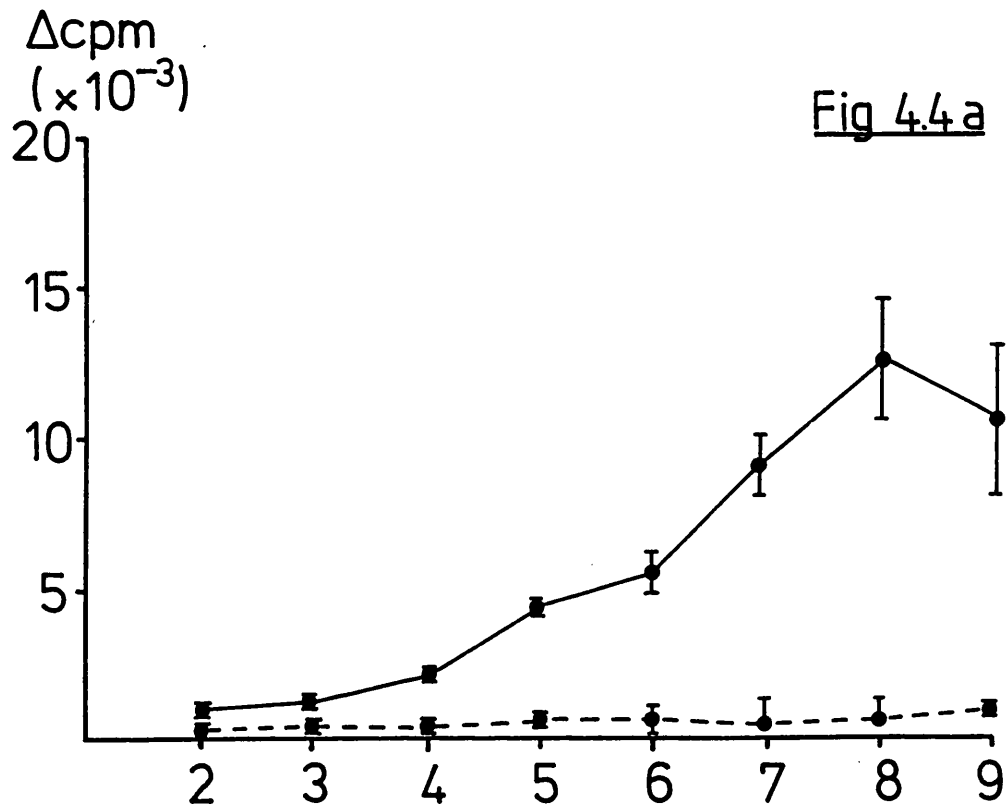
thereafter increased rapidly, reaching a peak value after 8 days in culture. Minimal incorporation of label by the T cell control was observed indicating the low, non-specific mitogenicity of this serum supplement. The variation between individual AMLR responses was very low up to day 7 of the culture period, but increased on days 8 and 9.

Figure 4.4(b) represents the AMLR proliferative responses observed in complete medium containing 10% (v/v) FCS. The response was apparent at an earlier stage in the culture period and increased at a greater rate than that observed for cells from the same population cultured in NU-SERUM. Also, the peak proliferative response attained was approximately 30% greater in the presence of FCS. Individual variations in the AMLR observed between quadruplicate cultures and between subjects were, however, substantially larger than for cultures containing NU-SERUM. The T cell control background incorporation was also greater, possibly reflecting the mitogenic qualities of the FCS.

..... AMLR proliferative responses for cultures in .....  
complete medium containing 10% (v/v) autologous

Figure 4.4 Kinetic AMLR proliferative response of T cells ( $1 \times 10^5$ ) to autologous, irradiated (2500 rad) non-T cells ( $1 \times 10^5$ ) when cultured in complete medium (see Materials and Methods p66) containing (a) 10% (v/v) NU-SERUM and (b) 10% (v/v) FCS. Each point represents the mean  $\pm$  s.e.m. of responses from cells of three healthy individuals:

The solid lines represent  $\Delta$ cpm AMLR responses; the dashed lines represent background incorporation for T-cell controls. Non-T cell controls incorporated insignificant levels of radioactivity.



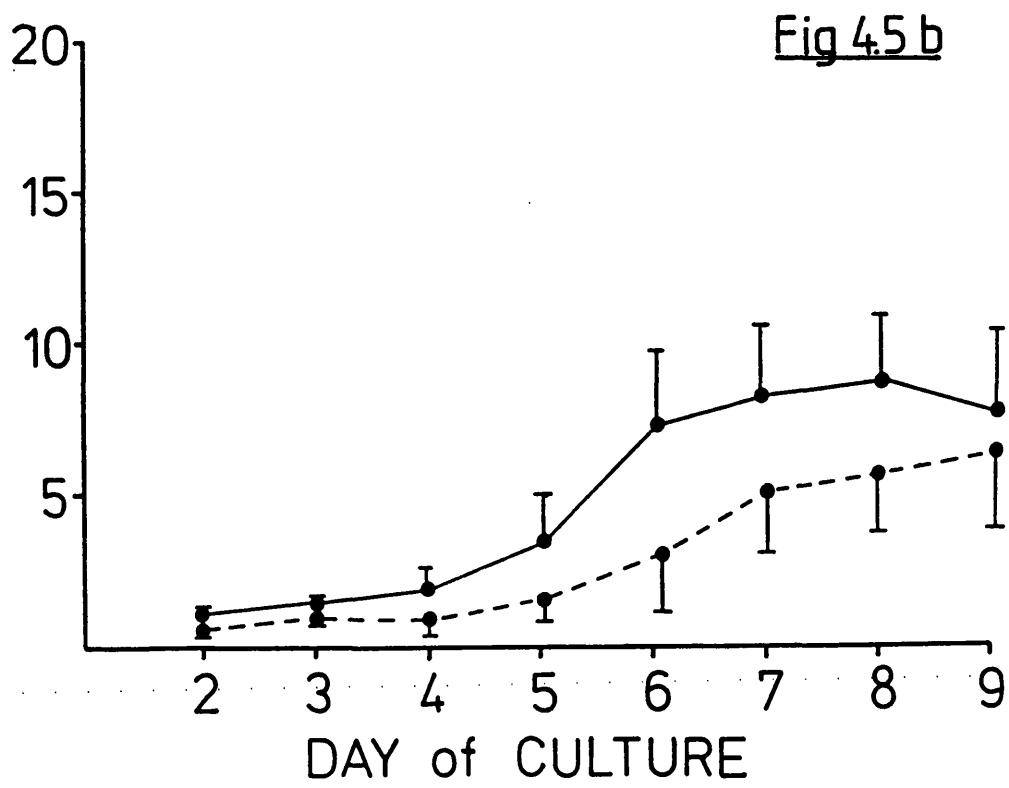
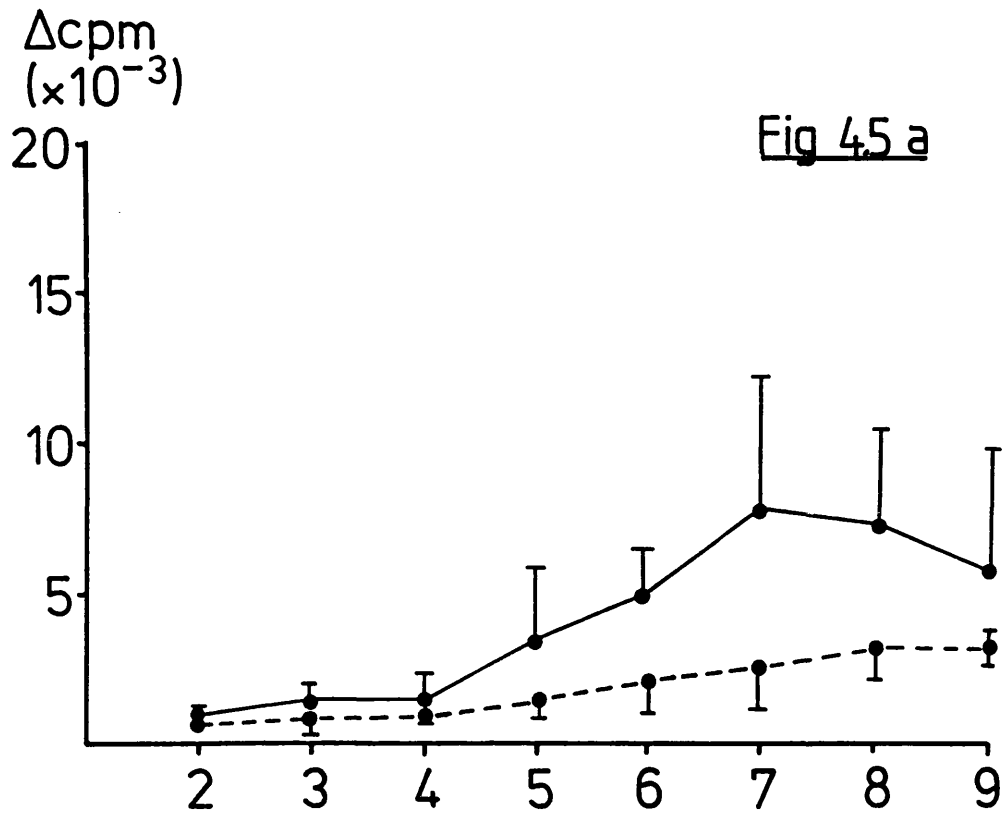
serum are shown in Fig 4.5(a). Responses varied considerably between the three individuals studied, the peak proliferative response occurring either on day 7 or day 8. The T cell control background incorporation steadily increased over the eight day period although the variation between these values was not large. The relatively high variation in response obtained between individuals appeared to be peculiar to the use of autologous serum. Whether this reflects different levels of total serum protein, a peculiar serum protein or the presence of a stimulatory or inhibitory factor was not determined.

The responses observed in the AMLR in the presence of pooled human AB serum are represented by Fig 4.5(b). The use of this serum supplement presented several problems. The heat inactivation process induced precipitation and flocculation of serum components, which necessitate centrifugation of the serum and may have affected its nutrient content. Also, batch to batch variability was increased because of the small volume of serum obtained.

The responses obtained for the AMLR using 10% pooled human AB serum supplement were generally

Figure 4.5 Kinetic AMLR proliferative responses of T cells ( $1 \times 10^5$ ) to autologous, irradiated (2500 rad) non-T cells ( $1 \times 10^5$ ) when cultured in complete medium (see Materials and Methods, p<sup>66</sup>) containing (a) 10% (v/v) autologous serum and (b) 10% pooled human AB serum. Each point represents the mean  $\pm$  s.e.m. of responses of cells from three healthy individuals:

The solid lines represent  $\Delta$ cpm AMLR responses; the dashed lines represent background incorporation for T-cell controls. Non-T cell controls incorporated insignificant levels of radioactivity.





similar to those observed with autologous serum, although the rate of growth appeared to be greatest between days 4 and 6 of the culture period, compared with a more gradual increase between days 4 and 7 in autologous serum. Background incorporation of the label into the T cell controls was significant after day 5 in culture and was also subject to wide variation in the case of pooled serum. The net AMLR response involves subtraction of the sum of the means of the cell control and the non-T cell control as described (Methods, p<sup>69</sup>). Because very little proliferation occurs in the irradiated non-T cell background control cultures, the net AMLR effectively depends upon the responses obtained in the AMLR cultures and the T cell control. Any manipulation of the culture conditions which acts to reduce the response of the latter will accordingly increase the net AMLR and increase the sensitivity of the assay. On these grounds, both human autologous serum and pooled human AB serum were considered unsuitable for the present purposes.

NU-SERUM and FCS were further investigated by using supplement concentrations of 2.5, 5, 10, 20 and 25% (v/v). Increasing concentrations of both of these culture medium supplements resulted

in concomitant increases in the degree of proliferation observed Figs (4.6 and 4.7). Although the AMLR proliferative responses observed for those cells cultured in FCS (Fig 4.6a) increased with increasing serum concentration in the culture medium, a parallel rise in the degree of proliferation observed in the background response was observed (Fig 4.6b), this being quite substantial at the highest serum concentrations. The responses observed in the presence of NU-SERUM (Fig 4.7a), also show an increase in proliferative response with increasing supplement concentration although not to the same extent as that observed with FCS. However, the background control responses increased relatively less compared to the AMLR response than in the case of FCS. The net proliferative response was as identifiable and distinct at the 10% (v/v) concentration as it was at higher supplement concentrations and replicate variation within an individual assay was smaller at this concentration than at any other concentration.

In view of all the above considerations, NU-SERUM at a concentration of 10% (v/v) was chosen for use as a supplement to the culture medium for routine investigation of the AMLR. As

Figure 4.6(a) The mean kinetic AMLR response for cells from three healthy individuals. T cells ( $1 \times 10^5$ ) were cultured with autologous, irradiated (2500 rad) non-T cells ( $1 \times 10^5$ ) in complete medium containing FCS at 2.5, 5, 10, 20 and 25% concentrations (v/v). (b) T cell controls obtained as described above except that irradiated non-T cells were replaced by irradiated T cells. No standard error bars are shown for reasons of clarity.

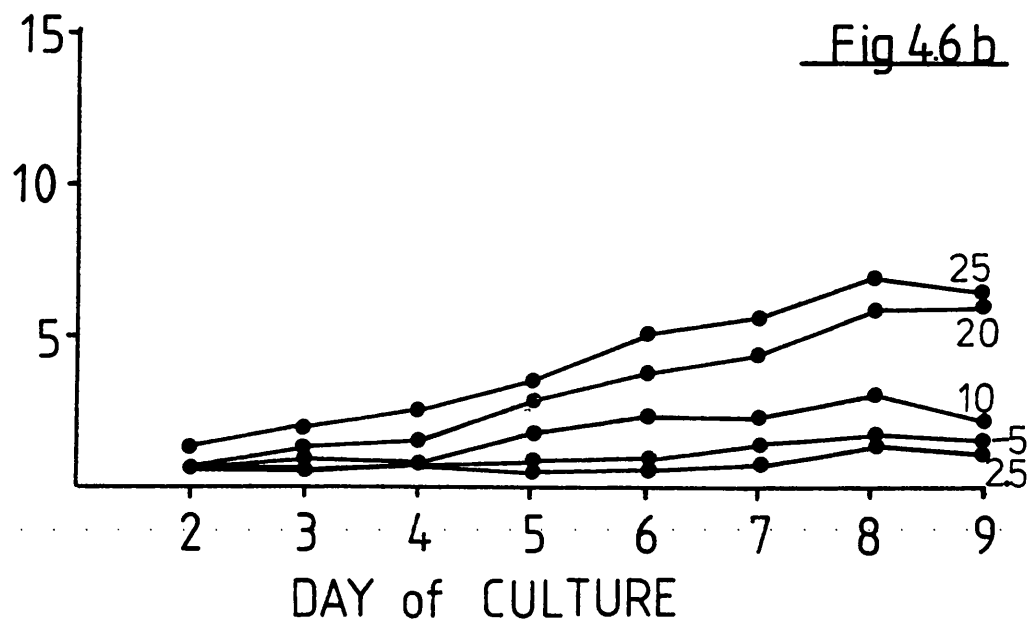
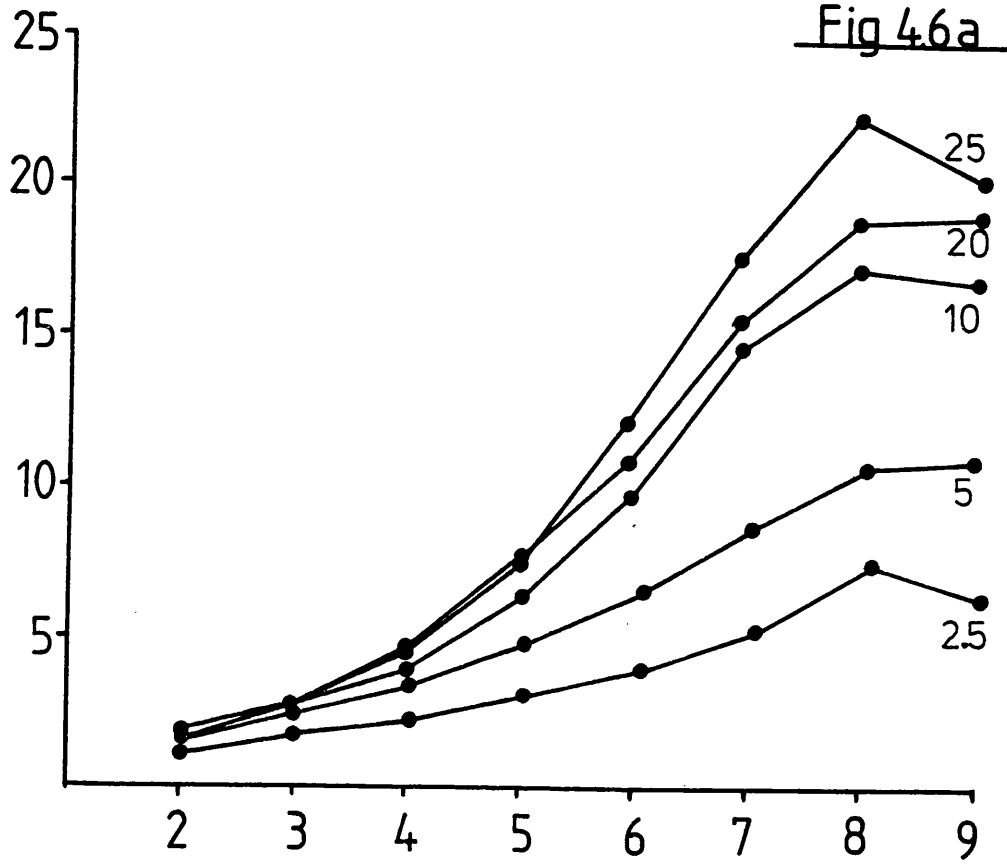
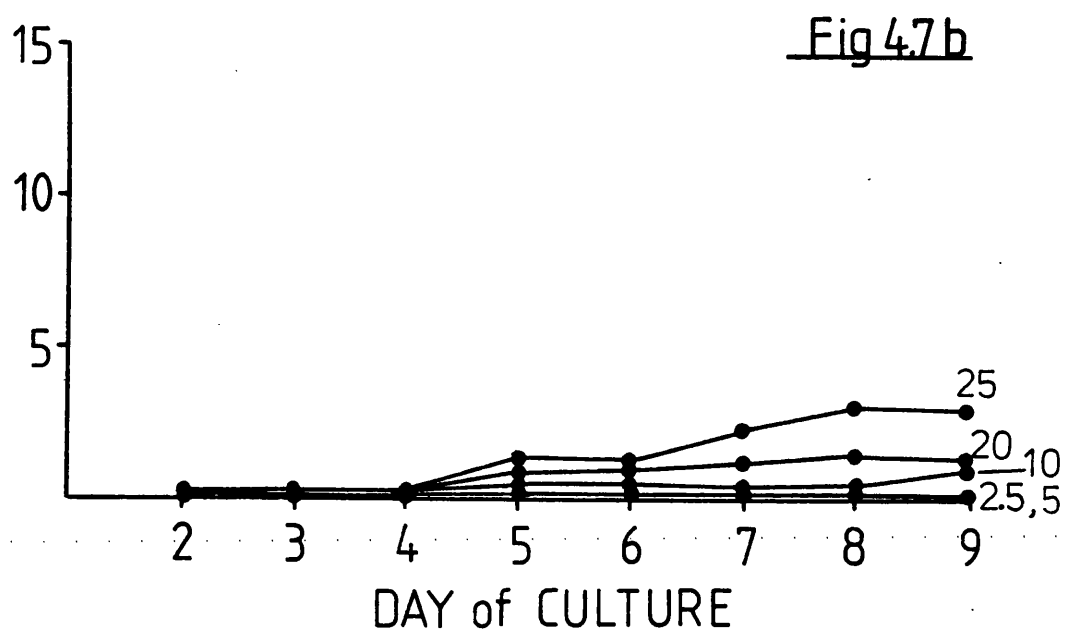
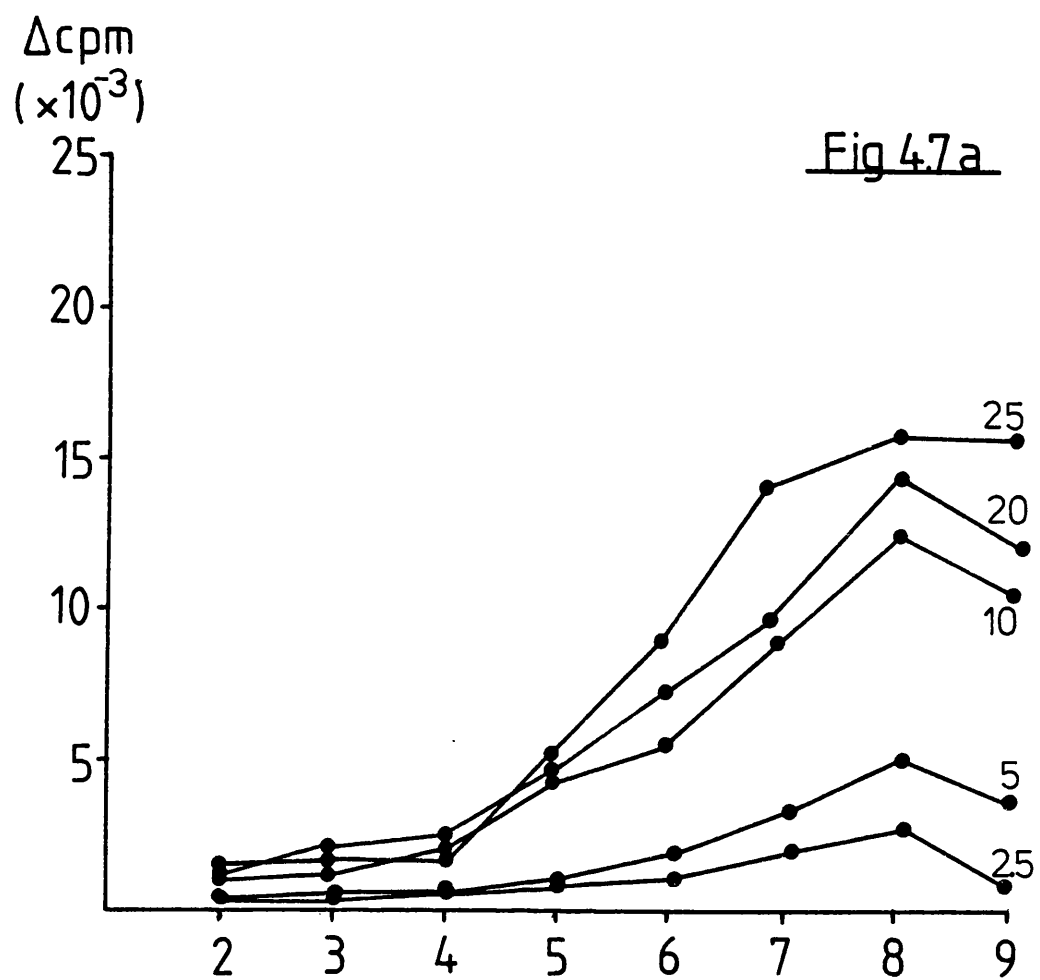
$\Delta \text{cpm}$   
( $\times 10^3$ )

Figure 4.7 (a) The mean kinetic AMLR response for cells from three healthy individuals. T cells ( $1 \times 10^5$ ) were cultured with autologous, irradiated (2500 rad) non-T cells ( $1 \times 10^5$ ) in complete medium containing NU-SERUM at 2.5, 5, 10, 20 and 25% concentrations (v/v). (b) T cell controls obtained as described above except that irradiated non-T cells were replaced by irradiated T cells. No standard error bars are shown for reasons of clarity.



NU-SERUM is a well-defined artificial serum supplement containing very low amounts (5%, v/v) of heat inactivated pooled human serum, batch to batch variability was minimal and heat inactivation before use was not necessary.

#### 4.6.2 Effect of cell number per well and ratio of T to non-T cells

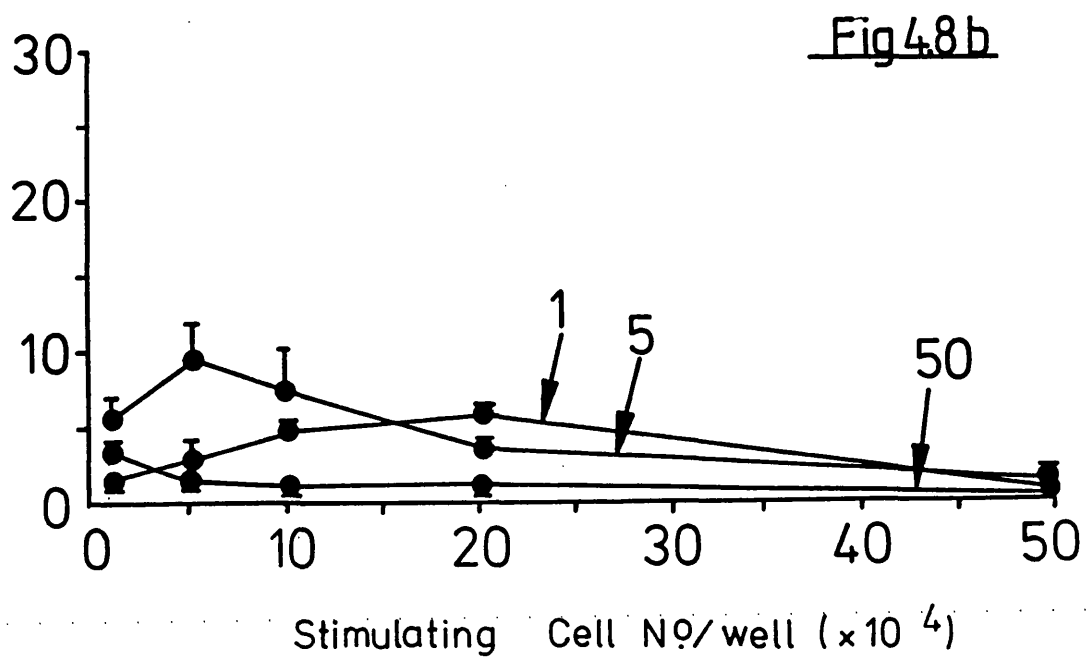
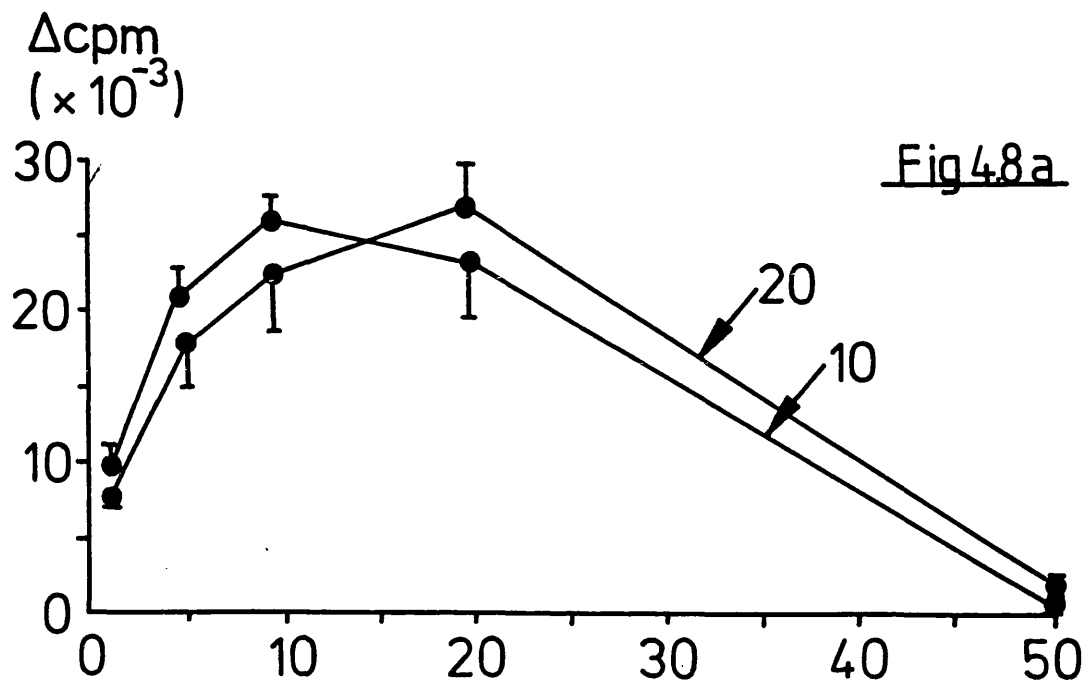
Fig 4.8 shows the variations in 7 day AMLR responses in 10% (v/v) NU-SERUM for different numbers of T cells in the presence of increasing numbers of non-T cells. In this way, the effects of both the cell number per well and the cell ratios could be studied simultaneously. Fig 4.8(a) shows the AMLR responses obtained for T cells at concentrations of  $1 \times 10^6$  cells/ml ( $10 \times 10^4$  cells/well) and  $2 \times 10^6$  cells/ml ( $20 \times 10^4$  cells/well) with varying concentrations of stimulating non-T cells. Both T cell concentrations respond initially in a dose-dependent manner, reaching peak proliferative responses at a 1:1 ratio of non-T cells. There was little difference in the magnitudes of the AMLR responses of the two T cell concentrations and the lower concentration was accordingly adopted as the standard protocol for all of the subsequent AMLR assays. Fig

Figure 4.8(a) The 7 day AMLR proliferative responses of fixed numbers of T cells ( $10$  &  $20 \times 10^4$  cells/well) to varying numbers of autologous, irradiated ( $2500$  rad) non-T cells. Each point represents the mean responses from cells of three healthy individuals.

Figure 4.8(b) The 7 day AMLR proliferative response of fixed numbers of T cells ( $1,5$  and  $50 \times 10^4$  cells/well) to varying numbers of autologous, irradiated ( $2500$  rad) non-T cells. Each point represents the mean responses from cells of three healthy individuals:

.....



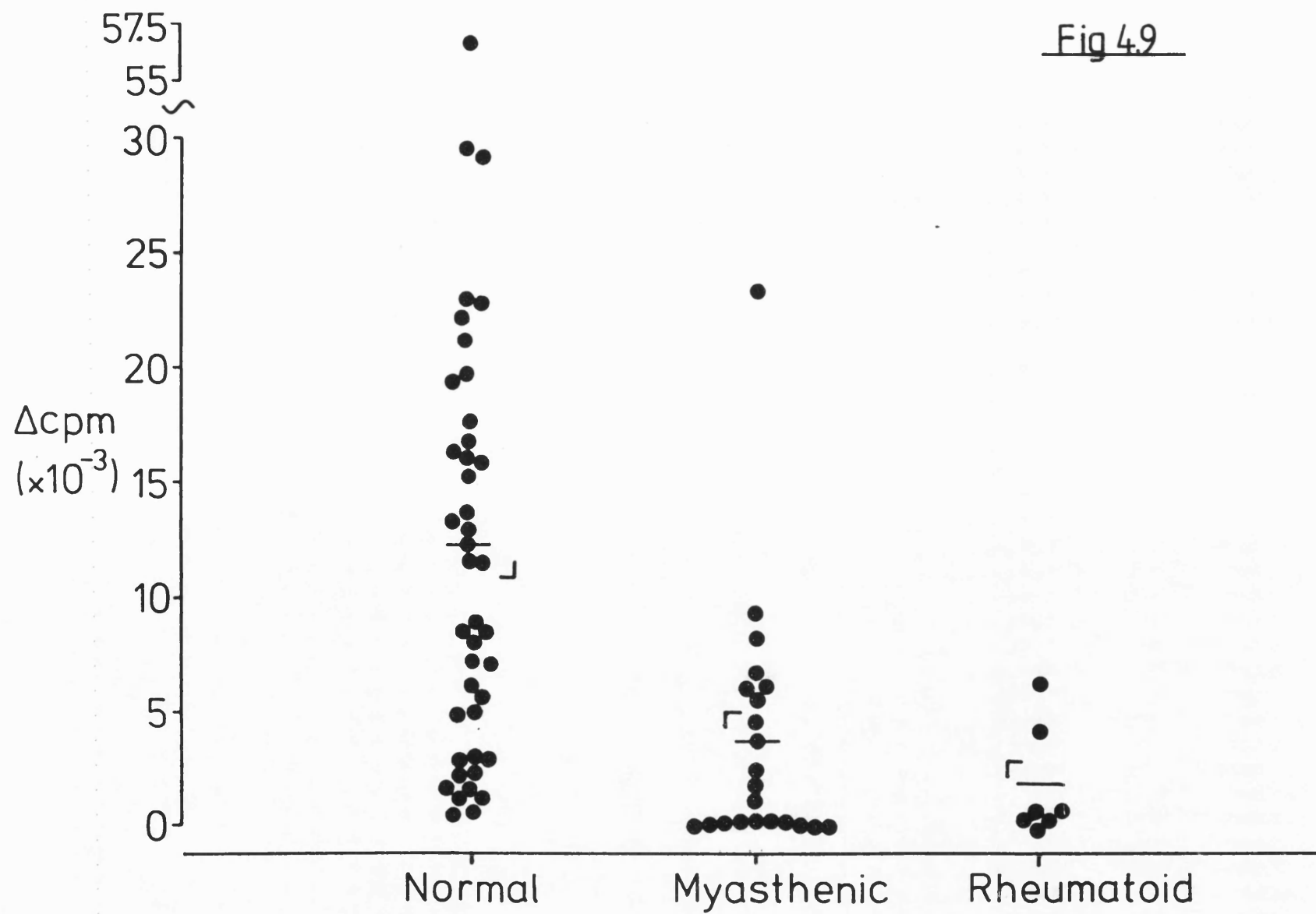


4.8(b) represents sub- and supra- optimum concentrations of T cells per well.

4.7. Peak AMLR proliferative responses for clinically healthy, myasthenic and rheumatoid individuals

Figure 4.9 represents the range of peak proliferative responses observed as the cpm, irrespective of the time point in the culture period on which it occurred, for 41 healthy individuals, 22 patients with myasthenia gravis, and 7 patients with rheumatoid arthritis. The responses for the healthy group have a range of 381 to 56384  $\Delta$ cpm ( $12228 \pm 1674$ , 41; mean  $\Delta$ cpm  $\pm$  sem, n) whereas those for the myasthenic patients have a range of 76 to 23052  $\Delta$ cpm ( $3622 \pm 1128$ , 22; mean  $\Delta$ cpm  $\pm$  sem, n). The difference between the mean responses of these two groups was highly statistically significant ( $P < 0.0001$ ) according to an analysis of the data by the Mann-Whitney test. Approximately 25% of the healthy individuals studied responded with a low magnitude, of less than 5,000 cpm. The control responses appeared to fall into defined classes which were low ( $< 5,000 \Delta$  cpm), medium (5,000-10,000  $\Delta$  cpm), and high ( $> 10,000 \Delta$  cpm) responses. This appears to be a real phenomenon as individuals who respond in a particular manner

Figure 4.9 The distribution of peak AMLR proliferative responses for cells from 41 normal healthy individuals, 22 myasthenic and 7 rheumatoid patients. The horizontal bar represents the mean of the group responses and the symbol ( $\Gamma/\sqcup$ ) represents the s.e.m.



tend to do so on repeat assay (see Section 4.14).

Fourteen (63%) of the twenty two patients with myasthenia gravis showed decreased responses in the AMLR whilst 7 of the patients responded in a manner similar to the medium range of the control AMLR response and one patient showed a high range control AMLR response. Of the seven patients studied here who had rheumatoid arthritis, 5 exhibited decreased AMLR and two responded in a manner similar to that observed for the medium range control-type responses. The range of responses observed in the rheumatoid group of patients was 53 to 5763  $\Delta$ cpm ( $1473 \pm 910$ , 7; mean  $\Delta$ cpm  $\pm$  sem, n), the difference between the mean response of this group and that of the control group was found to be highly statistically significant ( $P < 0.0001$ ) by the Mann-Whitney test.

#### 4.8. Kinetic analysis of the AMLR for the groups of healthy, myasthenic and rheumatoid individuals

All the responses shown in Fig 4.9 represent peak proliferative AMLR responses observed during the eight day culture period irrespective of when that response occurred. Each eight day AMLR culture in the study can be represented as a

profile, as shown in Fig 4.10 (a-e) which represent typical profiles for:

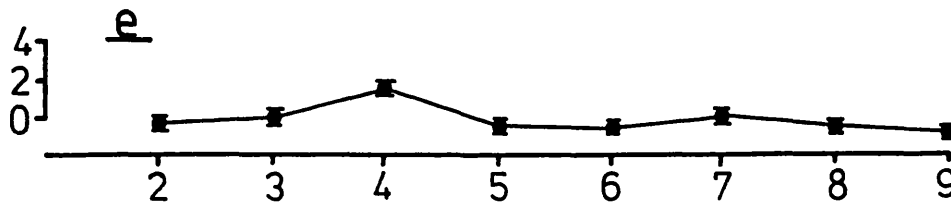
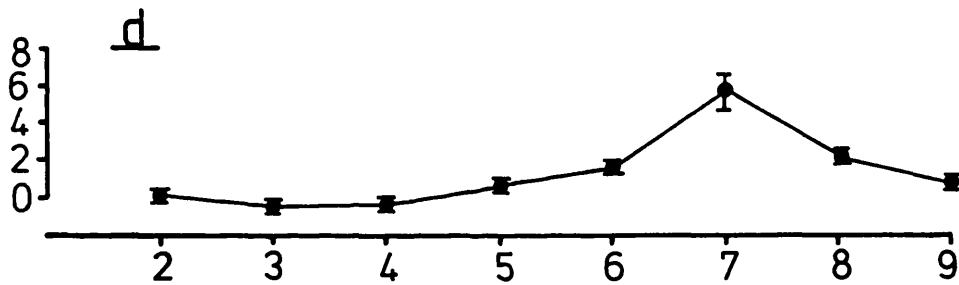
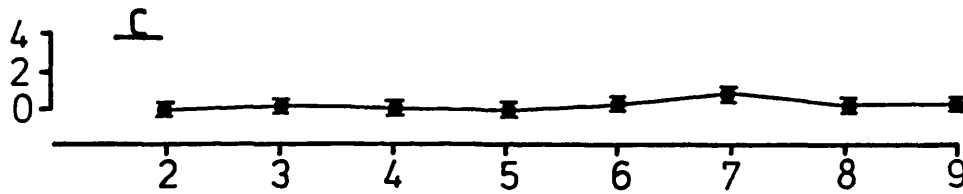
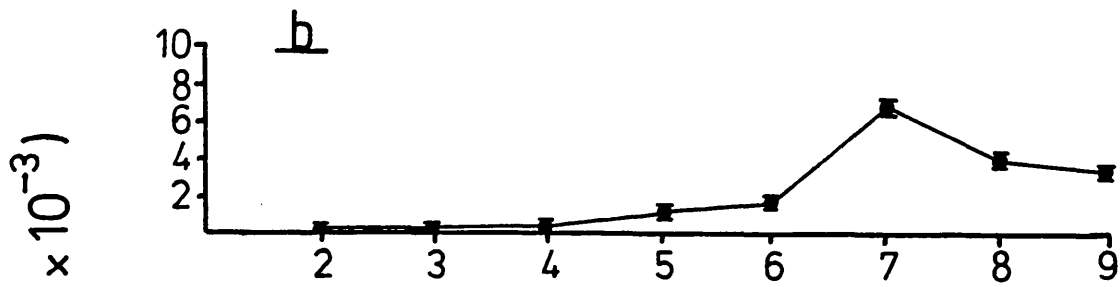
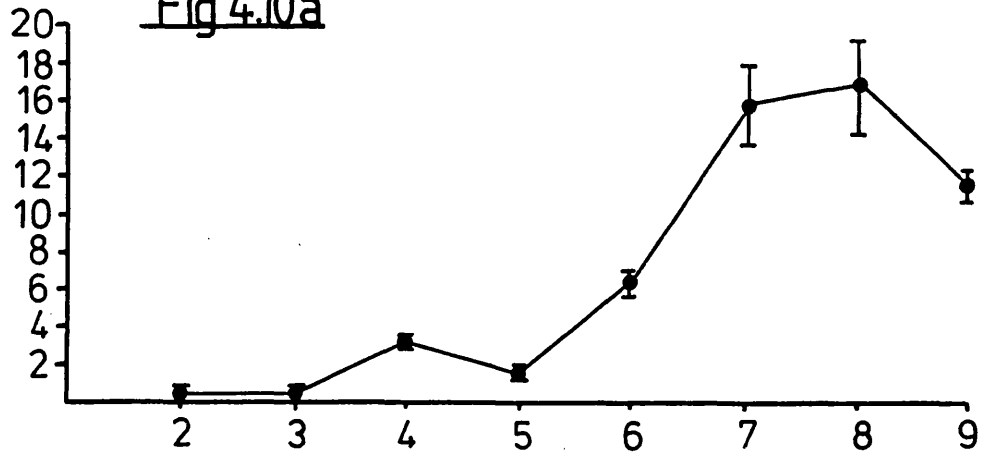
- a) normal individual
- b) high responding myasthenics
- c) low responding myasthenics
- d) high responding rheumatoid arthritis patients
- e) low responding rheumatoid arthritis patients

The curve shown in Fig 4.10(a) is from one of the high responder normal group ( $>10,000 \Delta\text{cpm}$ ), but is representative of the general trends shown by all of the normal responses. The AMLR response for the normal group of individuals becomes apparent after day 5 in culture and rises rapidly up to day 7. The proliferative responses generally reached a peak on day 7 or 8 in culture and thereafter decreased in magnitude. The fall in the proliferative response did not reflect depletion of nutrients as culture medium (100 $\mu\text{l}$ ) was replenished both on day 4 and day 7 of the culture period. The normal responses consistently showed small decreases after day 4 in culture. This was not due to the removal of endogenously produced growth factors as parallel cultures carried out for both day 4 and day 7, which did not undergo culture medium renewal, responded in exactly the same manner.

Figure 4.10 (a-e) Typical kinetic AMLR proliferative responses of normal, myasthenic and rheumatoid subjects. T cells ( $1 \times 10^5$ ) were cultured with autologous, irradiated (2500 rad) non-T cells ( $1 \times 10^5$ ) as described (Methods p 66) in complete medium containing 10% NU-SERUM. (a) The normal response of a clinically healthy individual; (b) A mid-normal type response of a myasthenic patient; (c) A low response of a myasthenic patient; (d) A mid-normal type response of a rheumatoid patient; (e) A low response of a rheumatoid patient.

Each curve is the response for a single individual but is considered to be representative and characteristic of a particular responding population.

Fig 4.10a



DAY of CULTURE



#### 4.9. The AMLR of MG patients

Two types of response generally observed for the group of patients with myasthenia gravis are represented by Fig 4.10(b) and 4.10(c). Clinical details and peak AMLR responses for this group of patients are presented in Table ~~4.4~~.

As previously discussed, the peak proliferative AMLR responses shown by the myasthenic group can be classed as low (14/22), medium (7/22), and high (1/22). The one myasthenic patient with a high range response showed an essentially normal AMLR profile of the type discussed above. Fig 4.10(b) represents a typical profile shown by the 7 myasthenic patients whose AMLR responses were in the medium range. The profile differs from the normal growth curve represented by Fig 4.10(a) in that little proliferation occurred before day 6 in culture. The peak response, occurring on day 7, was both distinct and short lived as this was generally followed by an equally rapid decrease in the proliferative response.

Figure 4.10(c) represents the responses observed by the 14 myasthenic patients who showed decreased or totally deficient AMLR responses. No

Table 4.4 The clinical details and proliferative responses, in the AMLR and to PHA-P and Con-A, of myasthenic patients.

\* Clinical status was assigned according to a modified Osserman (Osserman and Genkins, 1971) classification and any clear progression (i.e. exacerbation or remission) was noted.

\*\* AChE - anti acetylcholinesterase;

Thy - thymectomy;

Aza - azathioprin

Anti-acetylcholine receptor antibody titres were determined by radioimmunoassay.

TABLE 4.4 Proliferative responses in AMLR and to PHA-P and to ConA for myasthenic patients

PATIENT	AGE/SEX	CLINICAL STATUS*	TREATMENT**	DURATION OF DISEASE (YEARS. MONTHS)	ANTI-AChR ANTIBODY TITRE <sup>-10<sub>M</sub></sup> (x10 <sup>-10</sup> <sub>M</sub> )	AMLR ( Δ cpm)	PHA ( Δ cpm)	ConA ( Δ cpm)
1	25/F	IIb	AChE/Thy	0.7	570	8059	40000	N.D.
2	30/F	IIa	AChE	3.5	1288	5648	28130	N.D.
3	35/F	I	AChE	1.6	143	76	106200	N.D.
4	56/F	IIb/E	AChE/Thy	11.5	84	4332	37430	N.D.
5	51/M	I	AChE	3	56	106	31861	16077
6	52/M	I/R	-	5.7	25	23052	86358	25478
7	36/F	IIa/E	AChE	2.4	111	1801	103253	58553
8	29/F	IIa/R	-	2.8	N.D.	212	97261	40853
9	43/F	I	Thy	4.3	3.4	117	64498	52986
10	27/F	I	AChE	4.1	528	131	73878	52441
11	35/F	IIb/R	AChE	7.2	172	3842	115798	14020
12	38/F	I/E	-	8	N.D.	6539	80539	11928
13	49/M	IIa/R	-	3.3	28.6	494	31962	5542
14	41/F	IIa (diabetes)	AChE/Thy	3	3.5	2209	73798	N.D.
15	26/F	IIa/E	AChE/Thy/Aza	4	240	9472	25204	N.D.
16	40/M	I/E	-	0.5	N.D.	339	62106	N.D.
17	52/M	IIb/E	AChE	6.5	242	6250	75521	N.D.
18	67/F	IIa/R	AChE	3.5	256	111	48586	N.D.
19	42/F	I	-	1.9	0.7	83	N.D.	N.D.
20	33/F	IIa/E	AChE/Thy	4	137	5815	103022	55328
21	46/M	I	-	0.9	15	703	53200	N.D.
22	46/M	I	AChE	2.8	91	290	N.D.	1348

peak response was apparent and consequently the growth curve was flat. Despite this total unresponsiveness, the cells present in all of these cultures were responsive to the mitogens PHA-P and Con-A (see Fig 4.11), and greater than 95% of the cells were viable at the beginning of the culture period. Thus, the cells were capable of responding to certain types of stimuli but were unable either to induce an AMLR response or to respond in the AMLR.

#### 4.10. The AMLR of rheumatoid arthritis patients

Clinical details of the 7 rheumatoid arthritis patients studied are presented in Table 5 together with their peak AMLR responses. Fig 4.10(d) shows a representative AMLR growth curve for the two rheumatoid patients whose peak proliferative responses could be classed as being in the mid-range of the normal AMLR responses. The AMLR responses for these two patients were generally similar to those observed for the myasthenic patients who also responded with a mid-range normal AMLR response (Fig 4.10c).

Five of the seven rheumatoid patients studied showed very low AMLR responses similar to those observed in the low responding group of

Table 4.5. The clinical details and AMLR and mitogenic proliferative responses of rheumatoid arthritis patients. Clinical status was assigned according to standard clinical evaluation methods.

**TABLE 4.5** Proliferative responses in the AMLR and to PHA-P and to ConA for rheumatoid arthritis patients

PATIENT	AGE/SEX	CLINICAL STATUS	TREATMENT	DURATION OF DISEASE (YEARS. MONTHS)	CLINICAL DETAILS	AMLR (Δ cpm)	PHA (Δ cpm)	ConA (Δ cpm)
1	40/F	MILD	SALAZOPYRINE	2.0	SERO-POSITIVE (1:128)	5763	N.D.	34696
2	65/F	SEVERE	-	40.0	SERO-POSITIVE (1:64) EROSIVE, VASCULITIS	134	84987	8803
3	43/M	ACTIVE	-	7.0	SERO-POSITIVE NODULAR, EROSION. PLEURAL NODULES. PENICILLAMINE NEPHRITIS	-53	69342	26938
4	51/F	MILD	-	2.0	SERO-NEGATIVE	139	36240	13890
5	54/M	SEVERE	SALAZOPYRINE	1.0	SERO-POSITIVE EROSIVE	72	54029	6503
6	55/M	ACUTE	-	NEWLY DIAGNOSED	SERO-POSITIVE	64	126450	31346
7	55/F	MILD	-	2.0	IgG RF POSITIVE LATEX NEGATIVE	4089	N.D.	34275

N.D. = NOT DETERMINED

- : INDICATES NO TREATMENT

myasthenic patients. The degree of proliferation in the AMLR test cultures was similar to that observed in the corresponding background T cell controls and consequently, the cpm was minimal. The slightly increased response on day 4 indicates the possible initiation of an AMLR response but the response thereafter diminishes. This 'peak' on day 4 is not likely to represent a change in the kinetics of the AMLR response for this group of patients, but may represent the inability to sustain the response after this time. As in the case of the myasthenic group of patients, this apparent unresponsiveness was not due to reduced viability, as assessed by trypan blue dye exclusion at the beginning of the culture period and supported by mitogen responses.

**4.11. Mitogenic responses of purified T lymphocytes from healthy individuals and from patients with myasthenia gravis or rheumatoid arthritis**

The peak proliferative responses for purified T cells to the mitogens phytohaemagglutinin-P and concanavalin-A are presented in Figure 4.11. Because of the limited number of cells isolated from the peripheral

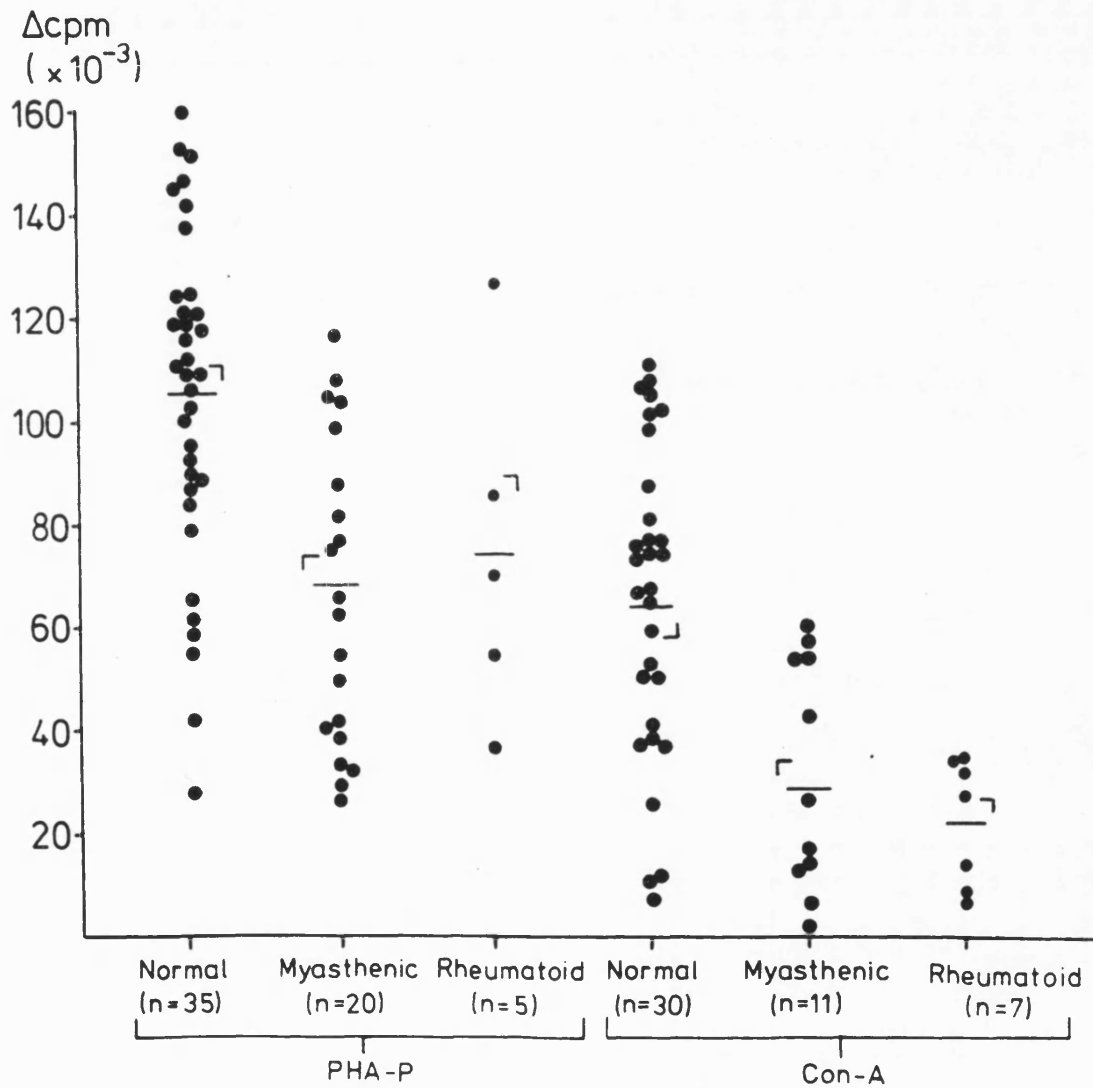
blood of various individuals, not all of the subjects whose AMLR responses were studied were included in this study.

The variation of the T cell response to PHA-P in the healthy group is quite considerable. The responses in this group range from 26204 to 158746  $\Delta$ cpm ( $103978 \pm 5474$ , 35; mean  $\Delta$ cpm  $\pm$  sem, n). The variation in the response to PHA-P for myasthenic subjects was also large, ranging from 25204 to 115798  $\Delta$ cpm ( $66930 \pm 6499$ , 20; mean  $\Delta$ cpm  $\pm$  sem, n) as was that for the rheumatoid subjects (36240 to 126450  $\Delta$ cpm;  $74209 \pm 15359$ , 5; range, mean  $\Delta$ cpm  $\pm$  sem, n). The differences between the mean responses of each of the two disease groups and that of the healthy group were statistically significant ( $P < 0.0001$  for MG,  $P < 0.0001$  for RA).

The proliferative responses to optimum concentrations of Con-A ( $10\mu\text{g/ml}$ ) were also studied on the same populations of purified T cells that were employed in the PHA-P study. As for the study of the PHA-P induced responses, the peak proliferative response to Con-A generally occurred on day 4, although variations of 1-2 days were occasionally observed. The responses for healthy individuals ranged between 5997-108999  $\Delta$ cpm ( $63310 \pm 5588$ , 30; mean  $\Delta$ cpm  $\pm$



Figure 4.11 Peak mitogenic responses of purified T lymphocytes ( $2 \times 10^5$ ) from normal, myasthenic and rheumatoid subjects to 0.2% PHA-P and 10 $\mu$ g/ml Con-A. The horizontal bar represents the mean response for each group and the symbol (L) represents the s.e.m. for each distribution.



s.e.m., n) and those for the myasthenic patients between 1348-58553  $\Delta$ cpm ( $30414 \pm 6619$ , 11; mean  $\Delta$ cpm  $\pm$  s.e.m., n). The range of peak proliferative responses to Con-A for the rheumatoid T cells was 6503-34696  $\Delta$ cpm ( $22350 \pm 4636$ , 7, mean  $\Delta$ cpm  $\pm$  s.e.m., n). The means of the proliferative responses for the myasthenic and rheumatoid group were both significantly different from that of the healthy group ( $P < 0.006$  and  $P < 0.001$  respectively).

#### 4.12. AMLR of healthy individuals: Associations between age, sex, and the immunological parameters studied

Previous investigators have reported conflicting findings regarding the effect of the age and sex of an individual on the AMLR response (Fournier and Charriere, 1981, Fernandez and MacSween, 1980, Moody et al., 1981). In this study, the associations between age, sex, peak AMLR response, peak PHA-P response, and peak Con-A response for 41 healthy individuals (25 males : 16 females) were investigated.

Fig 4.12 and 4.13 show that there is little correlation between most of the parameters studied. However, there is a significant positive correlation ( $P < 0.05$ ) between the peak

PHA-P response by the purified T cell population and the age of the donor subject (Fig 4.12b). This is also observed when the PHA-P data is log transformed. The use of these logarithmic transformations reveals a correlation between the peak PHA-P response and the peak Con-A response which was not apparent when the raw data were compared. Similar apparent correlation also occurs between log. transformed peak PHA-P data and raw AMLR data. The AMLR appears to be unaffected by the various parameters studied and any changes in the other immunological responses may reflect alterations in immunoregulatory mechanisms distinct from that of the AMLR.

#### 4.13. AMLR of patients with myasthenia gravis: Associations between age, sex and the immunological parameters studied

The degree of association between the immunological responses studied and the age, sex, anti-AChR antibody titre and duration of disease in the group of patients with myasthenia gravis was determined by correlation and regression analysis.

The correlation coefficients and statistical significance of these correlations are shown in

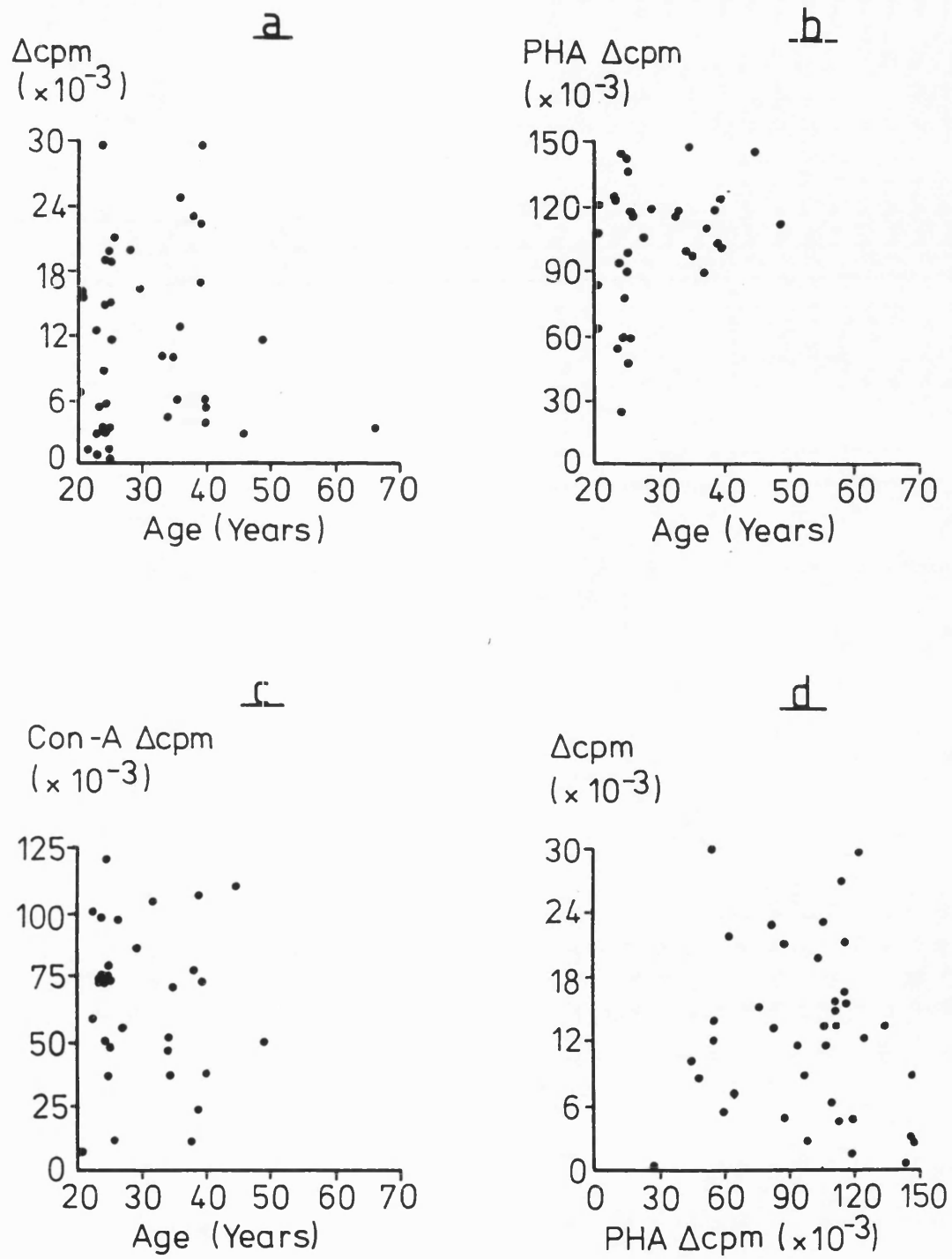
Figure 4.12(a-d) Correlations between various paired parameters for cellular responses from normal, healthy individuals:

(a) AMLR proliferative response versus Age.

(b) PHA-P induced T cell response versus Age.

(c) Con-A induced T cell response versus Age.

(d) AMLR proliferative response versus PHA-P induced T cell response.

Fig 4.12

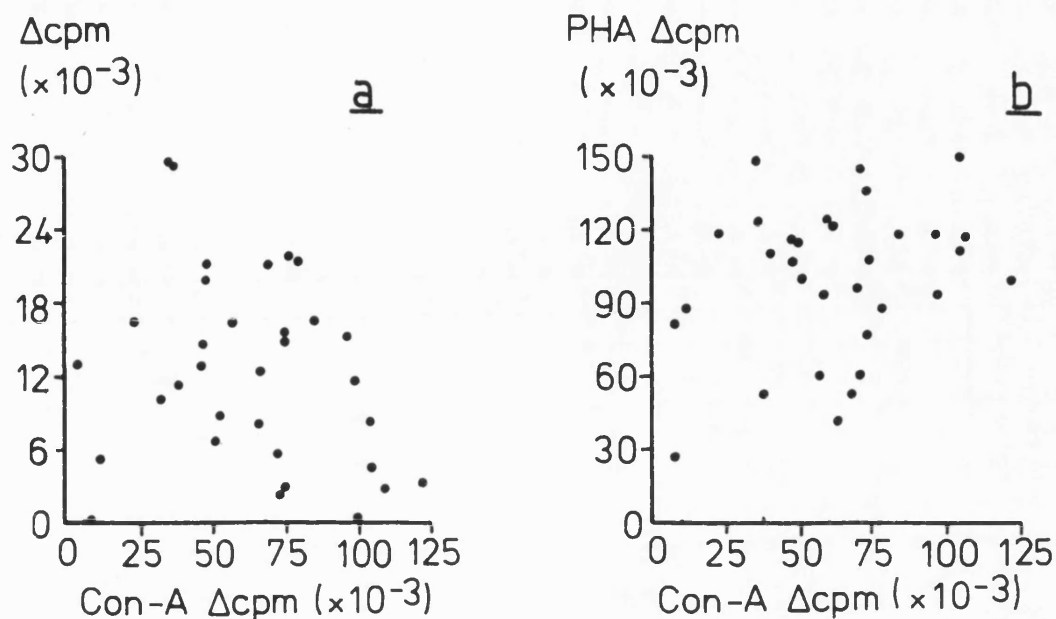


Figure 4.13 (a) The degree of correlation between the AMLR proliferative response ( $\Delta \text{cpm}$ ) and the Con-A induced T cell response for normal, clinically healthy individuals.

Figure 4.13 (b) The degree of correlation between the PHA-P induced T cell response and the Con-A induced T cell response for normal, clinically healthy individuals.

Table 4.6. No correlation was found between any of the parameters tested. Only three of all the pairs of associations tested showed significance values less than 0.1 but these were always greater than 0.05 and so were not considered to be significant. These three associations were PHA-P vs Con-A (slight positive correlation), Con-A vs age (slight negative correlation), and titre vs age (slight negative correlation). A positive correlation may be expected between PHA-P response and Con-A response although, as the two mitogens may stimulate separate or overlapping cell populations, the degree of correlation may not be high. The slight negative correlation between Con-A response and age may reflect an age-related loss of Con-A induced T suppressor cells and may account for the general increase in autoimmune states observed with increasing age. The slight negative correlation observed between the titre of anti-AChR antibody and age may be due to the existence of autoaggressive states, represented by increased humoral activity, which later diminishes as the whole immune system enters into the final stages of the autoimmune process. However, as the significance values are not less than 0.05, these associations cannot be interpreted as being important although their lack of significance may

Table 4.6 Correlation matrix of the parameters studied for the group of myasthenic subjects. None of the correlation coefficients shown was statistically significant unless stated otherwise.

	PEAK AMLR	PEAK PHA-P	PEAK Con-A	TITRE	AGE	DURATION
PEAK						
AMLR	***					
PEAK						
PHA-P	0.028	***				
PEAK						
Con-A	-0.086	0.337	***			
Titre	0.103	-0.335	0.338	***		
AGE	-0.021	-0.212	-0.578	-0.452	***	
						(0.10 < P > 0.05) (0.1 < P > 0.05)
DURA-	0.306	0.057	-0.133	-0.078	0.291	***
TION						



be due to the relatively small population number studied.

#### 4.14. Reproducibility and variation of the normal AMLR response - a longitudinal study

The AMLR of ten healthy individuals (6 males, 4 females) of age range 22-40 years, were studied at least three times and up to 14 times over a period of 3 years. Figure 4.14 shows the range of responses with the mean response and s.e.m. for each of the ten individuals. Large variations in the response may be observed for several of the individuals which may not necessarily be due to experimental variation. Fig 4.15 shows the response observed for two individuals studied over a period of three years and suggests that a seasonal variation in the AMLR response may account for the intra-individual range observed in Fig 4.14. Although the data required to construct a complete picture of the possible variations that may occur over a 12 month period were not available, there are indications that some type of cyclical response may be occurring, possibly involving a peak of activity during the summer months. Although this presents an intriguing possibility, it is clear that a meaningful

Figure 4.14 The range of peak AMLR responses measured over a period of three years in 10 normal, healthy individuals. Sex (M/F) and age of subject are noted on the abscissa. The horizontal bars represent the mean of the responses for each individual and the symbol ( $\Gamma$ ) represents the s.e.m.

$\Delta\text{cpm} (\times 10^{-3})$

Fig 4.14

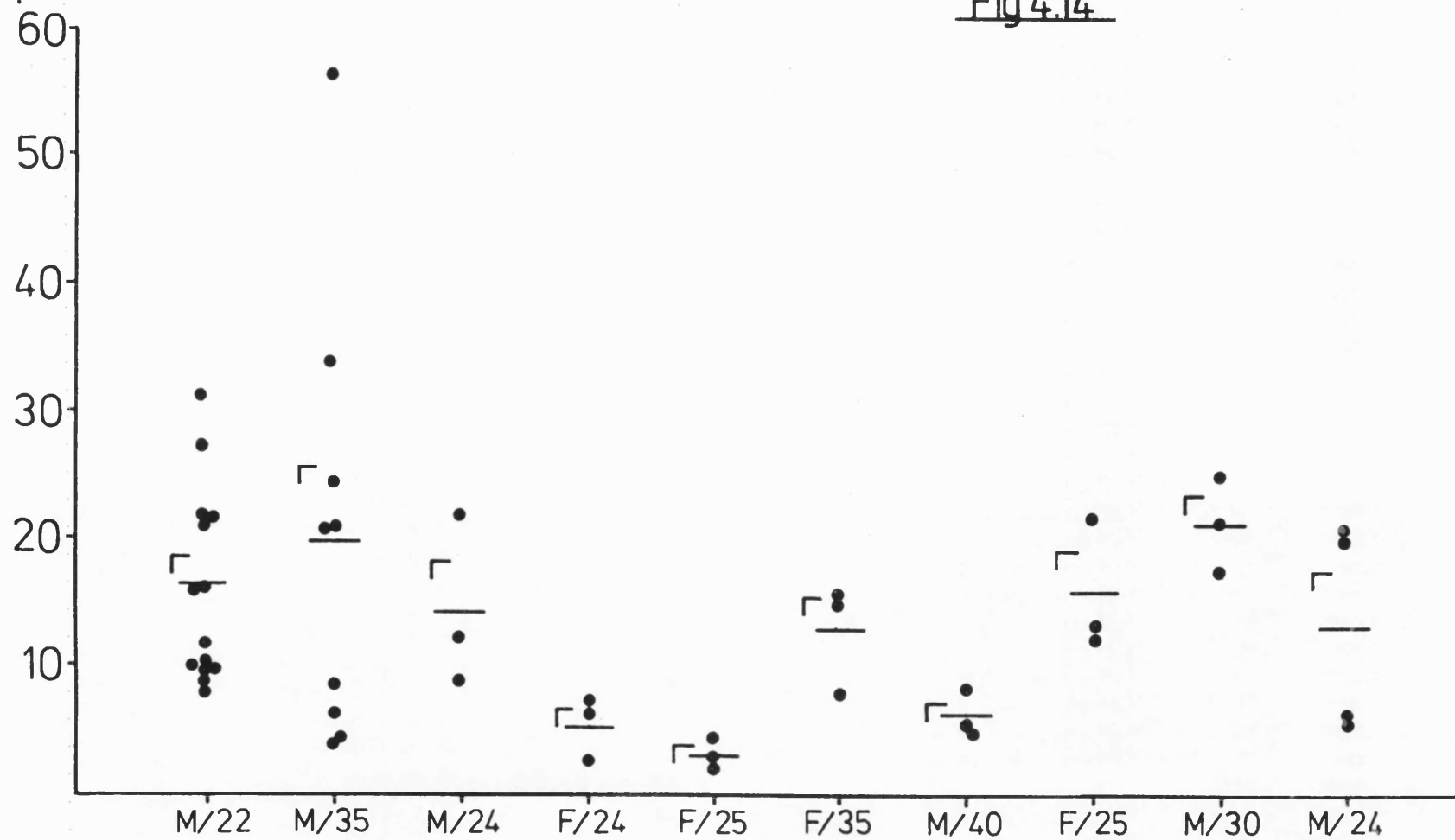
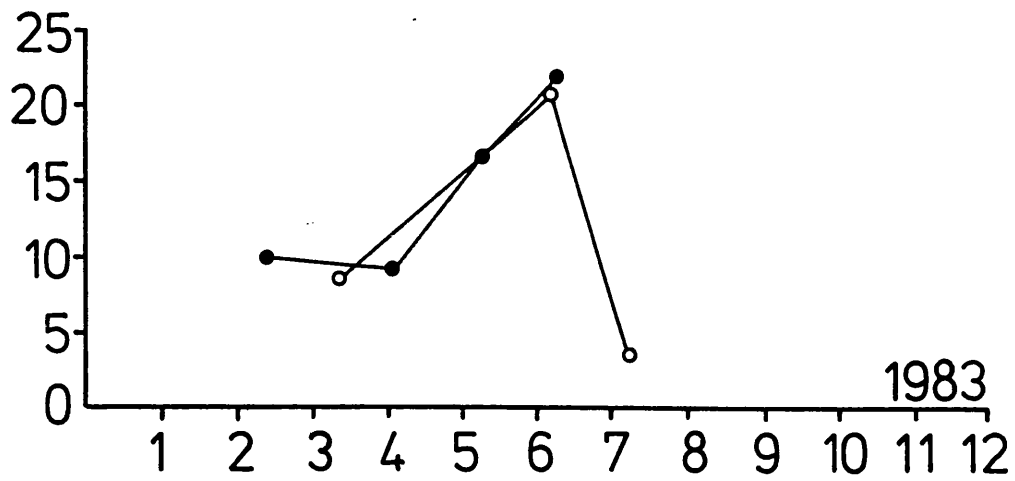
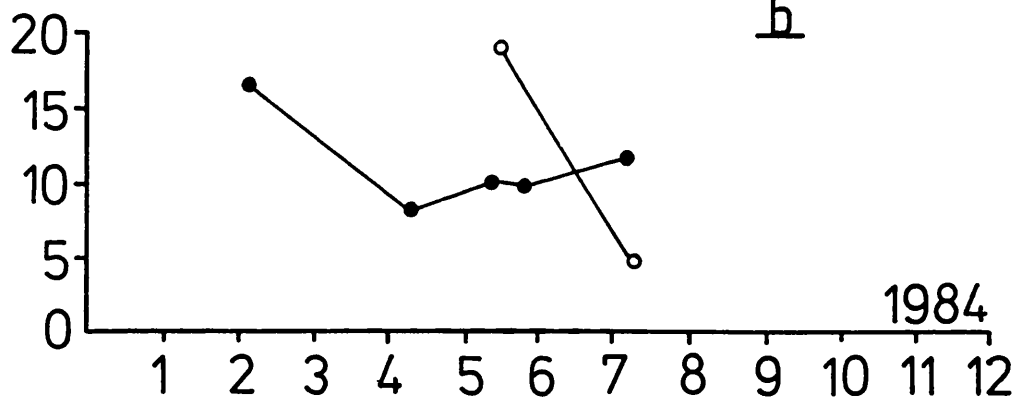
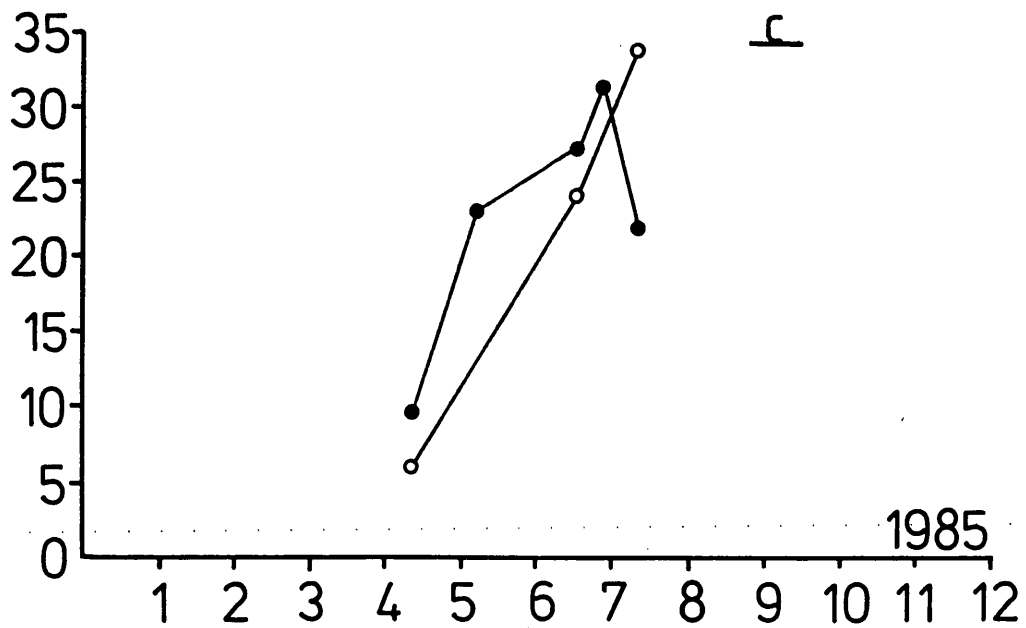


Figure 4.15 (a-c) The variation in the peak AMLR response for two clinically healthy male individuals studied over a period of three years. The closed circles ( ● — ● ) represent the responses of a 22 year old male and the open circles ( ○ — ○ ) represent the responses of a 35 year old male. Month 1 represents January of the year and month 12 represents December of that year.

Fig 4.15 a $\Delta\text{cpm} (\times 10^{-3})$ bc

Month

investigation would involve much more extensive studies than were possible in the context of this thesis.

## 5. DISCUSSION:

The establishment of standard culture conditions is fundamental for any comparison of the AMLR in normal individuals and in myasthenics. A comprehensive study of the factors affecting the AMLR in normal individuals was carried out by Ng and Russell (1982), who examined the AMLR with respect to duration of culture, the type and concentration of stimulating cells and the day-to-day variation in the results. The results obtained in the present study will be compared with those of Ng and Russell (1982), and also with those of other authors.

Although the responding cell type in the AMLR is agreed to be a T lymphocyte (Opelz et al., 1975; Kuntz et al., 1976; Sakane et al., 1978), controversy remains over the identity of the stimulating cell. The conflicting results obtained, showing maximum proliferation using different stimulating cell populations, may be due to the different methods of cell purification and cell classification used in various studies (Beale et al., 1980). The study of Smolen et al., (1982), (see Introduction, p 20 ), supported the view that more than one type of

stimulating cell exists. In an attempt to avoid the complications generated by further fractionating both the T cell and Non-T cell populations, the AMLR culture conditions were established by using whole T-cell and whole non-T cell populations. The various aspects of the culture conditions that were studied were:-

- i) The fractionation method used for separating T cells and non-T cells.
- ii) The effects of various serum supplements.
- iii) The effects of cell number per culture.
- iv) The effects of the ratio of T cells to non-T cells.
- v) The length of the incubation period.

All of the aspects outlined above were studied using PBL isolated from normal, healthy individuals.

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### 5.1. The method of T cell/non-T cell fractionation

Although no attempts were made to identify the stimulating cell within the non-T cell population, an attempt was made to ensure that the fractionated T and non-T cell populations were as highly purified as possible. Analysis of cell surface markers and responses to the mitogens Con-A and PHA-P were used as indicators of the degree of purity attained in each cell population following separation. The two general methods of purification tested were nylon wool passage and rosette formation with density gradient centrifugation. Each method depends on the presence of a particular surface marker. Nylon wool selects for B cells expressing immunoglobulin on their surface (Julius et al., 1973) while rosette formation, using SRBC, depends on the presence of a SRBC receptor on T lymphocytes (Coombs et al., 1970).

The results generated in this thesis indicate that E-rosette separated T and non-T cells yielded cell populations of significantly higher purity than that achieved by using nylon wool passage. Further, treatment of the SRBC with the sulphydryl compound, 2-AET, or with neuraminidase, prior to their use in a rosetting

procedure yielded rosettes which were both larger and more stable. This observation was in agreement with those of Kaplan and Clark (1974) and of Madsen and Johnsen (1979) who described the SRBC<sub>AET</sub> technique as 'effective, reliable and time saving'.

Inherent in any separation and isolation procedure is the possibility of challenging the lymphocytes with xenoantigens. This was discussed in the Introduction in terms of whether the AMLR is a true phenomenon, or is the result of xenoantigenic challenge. However xenoantigens affect the AMLR, the method used for separating the cells may influence this contribution. Thus, nylon wool (being a polyamide) could be considered to provide an excellent polymeric antigenic structure for the direct activation of B cells (by cross-linking surface receptors) and possibly even T cells and macrophages. Further, if E-rosette separation methods are employed, it is possible that the isolated T cells are activated during the rosetting procedure by the treated SRBC.

Whatever the role of xenoantigen in the AMLR, a significant difference was observed between the AMLR in normal individuals on the one hand and

in myasthenic and rheumatoid patients on the other. Thus, in the patients with autoimmune diseases, a change in a particular cell subset may have occurred resulting in the deficient autologous, or deficient anti-xenogeneic, response observed. All that can be claimed for the present work is that it has attempted to keep experimental manipulations of the cells used in the AMLR to a minimum, and hence reduce the effects of xenoantigenic contact.

#### 5.2. The effects of serum supplement type

The presence of xenoantigens in media may also be regarded as another source of proliferative stimulus for the AMLR. These xenoantigens may be present in the separation media (during isolation procedures) or in the culture media. A precautionary note was sounded by Hellman and Stobo (1982), who suggested that the specificity of the AMLR assayed in culture medium containing FCS is different from that assayed in either autologous or heterologous serum. More recently, xenoantigens have been suggested as, at least, augmenting (MacDermott and Bragdon, 1983), and, at worst, causing the response (Huber et al., 1982; Kagan and Choi,

1983).

The data presented in this thesis showed that the use of FCS resulted in a greater degree of cell proliferation in the AMLR than that observed with autologous, pooled AB, or NU-SERUM. It is possible, however, that FCS was acting in a mitogenic manner, as both the AMLR and T cell control responses were increased. The use of NU-SERUM, a well - defined low serum protein supplement, yielded AMLR responses of similar magnitude to those observed with complete medium containing FCS, but with low T cell control responses and was consequently preferred. NU-SERUM is a commercially available serum substitute containing a number of growth factors, vitamins, hormones, trace elements and various cell nutrients. It has been compared favourably with FCS, in terms of lower batch-to-batch variation and less mitogenic qualities, when used in the murine allogeneic MLR (Gibb et al., 1985).

NU-SERUM was also shown here to support the AMLR to a greater extent than either autologous serum or pooled human AB serum, while yielding lower background T cell control responses. The background responses observed in the presence of autologous serum and pooled human AB serum appear

to follow the proliferative responses observed for the AMLR, albeit in a diminished manner. These gradually increasing responses may have been due to particular stimulatory constituents of the serum samples, and did affect the net AMLR observed in most cases.

Thus, NU-SERUM was chosen for routine analysis of the AMLR. The ability to obtain a supply of culture medium supplement of low batch-to-batch variability, low non-specific mitogenicity, yielding satisfactory AMLR responses at a relatively low cost enabled the normal AMLR to be investigated in a controlled manner, with reasonable expectations that the response can be attributed to the stimulator cells, rather than to non-specific mitogenic substances present (Gibb et al., 1985) in serum.

### 5.3. The effects of cell number and the ratio of T cells to non-T cells per culture

Fundamental to the elucidation of the interactions that occur in the AMLR, is the observation by Opelz et al., (1975) that the amount of [ $^3\text{H}$ ]-Tdr incorporated by unfractionated lymphocytes in culture can be dramatically increased by increasing the ratio of T and non-T

cells; an observation confirmed by Ng and Russell (1982).

In the present study, an increase in the proliferative response was observed with increasing numbers of unfractionated non-T cells.

Among the five concentrations of responding T cells tested, the assays using  $1 \times 10^5$  and  $2 \times 10^5$  T cells/well showed the highest dose-dependency on non-T cell concentration, and, in both cases, peak responses occurred with 1:1 ratios of T to non-T cells. Assays involving either high or low concentrations of T cells resulted in very low proliferative responses.

Most of the data published to date use cell concentrations of  $2 \times 10^5$  cells in a total volume of 0.2ml culture medium, and cell ratios of 1:1 (ie  $1 \times 10^5$  T cells and  $1 \times 10^5$  non-T cells). These conditions were used here and combined the effects of maximum response with efficiency of cell usage. In contrast to these findings, both Kuntz et al., (1976), and Kalden et al., (1983), reported optimum proliferative responses using stimulating cell to responding cell ratios of 2:1. Gupta (1984), on the other hand, carried out AMLRs in which B cells and macrophages were used

as stimulating cells, over a range of T cells to non-T cells ratios of 1:8 to 1:1 and found that equal numbers of cell types yielded the maximum response, although no higher T cell to non-T cell ratio was investigated.

Low proliferative responses observed at non-optimal cell concentrations and ratios may result from the effects of substrate exhaustion, toxic metabolites, altered pH at higher cellular concentrations or excessive or inadequate cell-cell contact, as proposed by Knight (1982).

Knight (1982) emphasised that the proximity of cells in culture, culture vessel shape, proportion of responding to stimulating cells, and the length of the culture period may all play important roles in the observed responses of many assays involving lymphocyte proliferation.

It is also possible that high responses to stimulation may occur within a narrow range of doses of stimulant and only at certain times during the culture period (Ling and Kay, 1976).

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#### 5.4. The length of the culture period

In the majority of studies which have investigated the AMLR in normal, healthy individuals and in patients with autoimmune diseases, the response has only been determined on one day, usually day 6 of the culture period. This has been the case, with few exceptions, since Kuntz et al., (1976) followed kinetics of the AMLR over a nine day period, and observed the greatest stimulation index on day 6.

The comprehensive study by Ng and Russell (1982) of 30 normal subjects showed that the maximal response was evenly distributed between days 6, 7 and 8 of the culture period; for the majority (26) of the subjects studied. The present study noted that the peak responses occurred on the eighth day and less frequently, on the seventh day of the culture period.

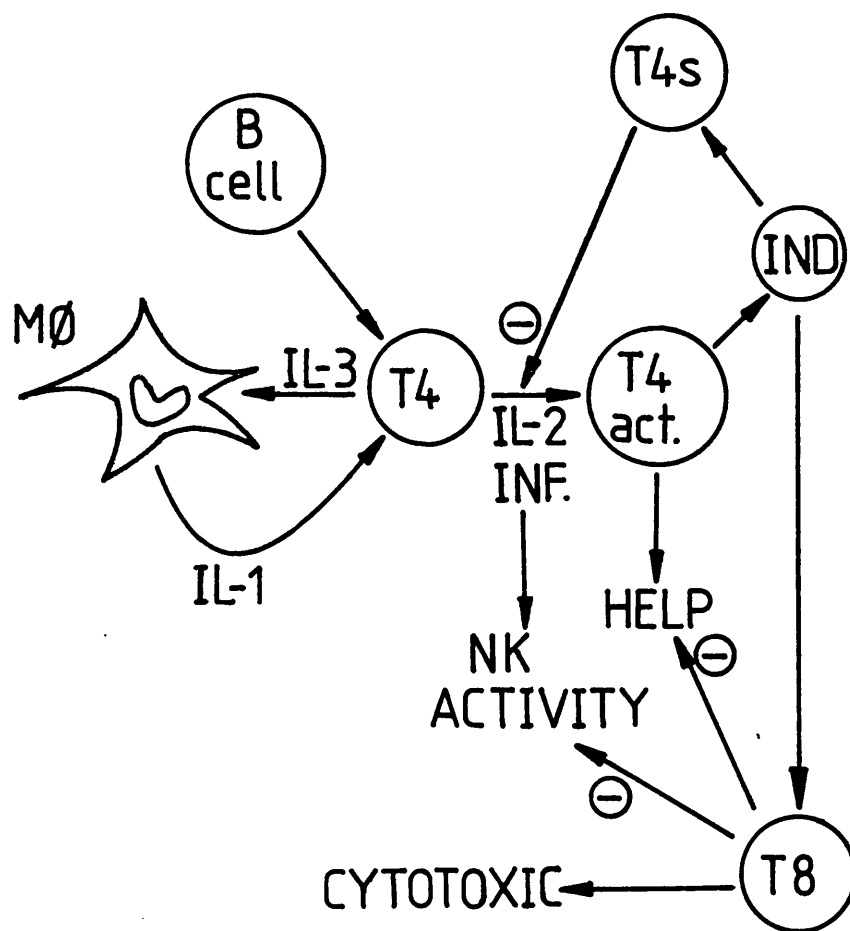
Other studies that have noted the peak AMLR response from kinetic investigations were based on results from low numbers of normal subjects. Miyasaka et al., (1980) noted a peak AMLR response on day 7 of a nine day culture period for five normal subjects, whilst Pope et al., (1984) suggested that the maximal AMLR response occurred



Fig 5.1. A schematic representation of the possible cellular interactions that may occur in the AMLR.

Initial macrophage or B cell stimulation of T4 cells through Ia antigens may result in the activation of these T4 lymphocytes. Release of IL-2 may then act to stimulate T4 inducer cells to activate both T4 suppressor cells and T8 suppressor cells. The T8 suppressor cells may act by suppressing T4 cell help in Ig synthesis and may suppress natural killer cell activity. Cytotoxic T8 cells may also be generated. T4 suppressor may negatively feedback and suppress T4 cell activation. Increased Ia antigen expression or foreign antigen processing by macrophages may result in increased T4 activation.

Key:	MØ	macrophage
	T4 act	Activated T4 lymphocyte
	IND	T4 Inducer cell
	T4 <sub>s</sub>	T4 Suppressor cell
	NK Activity	Natural Killer cell activity
	IL-1,2,3	Interleukin 1,2,3
	INF	Interferon
	⊖	Suppression

Fig 5.1

on day 7 of a seven day culture period, using only two normal subjects. Further, Ransohoff and Dustoor (1983) claimed that the peak AMLR response occurred on day 6 of a ten day culture period. However, no data points were recorded for days 7, 8 or 9, which must cast doubt on such conclusions.

The results obtained by Ng and Russell, (1982), on 30 normal subjects, would appear to be, in part, substantiated by the results presented in this thesis on 41 normal subjects. These observations may form the basis for future investigations of the normal AMLR, and enable the appropriate comparisons to be made correctly.

Apart from identifying the true peak response, a kinetic profile yields further information about the AMLR. Differences in the kinetics of the AMLR have been observed between the various connective tissue diseases (Laffon et al., 1983) and in primary biliary cirrhosis (James et al., 1980). Laffon et al., (1983) suggested that analysis of the day to day kinetics of the response gave information about differences in immunoregulatory circuits. Indeed, if the AMLR had only been measured on day 7 in the study of Laffon et al., (1983), early low responses in some disease group subjects

would have been missed.

Although no gross changes in kinetics for the AMLR responses were observed in the present study of myasthenic or rheumatoid subjects, it was evident that the responses did not occur until much later in the course of the culture period for the disease groups, when compared to the responses of the normal subjects. These observations further support the view of Ng and Russell, (1982), that the results obtained in the AMLR should be expressed in terms of the peak response based on a variable duration of culture.

#### 5.5. The variation in the normal AMLR

Using a similar stimulating cell population (ie unfractionated non-T cells) to that employed in the present study, Ng and Russell (1982) observed that, in some subjects, the time to peak AMLR responses and the magnitude of that response varied significantly, even when blood samples were obtained on consecutive days from the same individual. However, despite the variability of response patterns and the time to peak response, the magnitude of the maximal response was shown to be relatively reproducible with blood taken on

consecutive days in 6 normal subjects (Ng and Russell, 1982). Further, the reproducibility of the response was markedly improved when non-adherent non-T cells rather than unfractionated non-T cells were used as stimulators.

The data presented in this thesis are in accordance with previous reports (Sakane et al, 1978; Dock and Davey, 1980; Ng and Russell, 1982), which showed a wide range of AMLR responses amongst normal controls. Using unfractionated non-T cells as the stimulating fraction, Ng and Russell (1982) showed that the maximal response of 30 normal individuals ranged from 400  $\Delta$ cpm to over 18,000  $\Delta$ cpm, with a mean of 5,700  $\pm$  4900. In the present study, a range of 381  $\Delta$ cpm to 56,384  $\Delta$ cpm was found, with a mean of 12,228  $\pm$  1674, for 41 normal subjects. This higher range and mean peak AMLR response may be a consequence of a different control population, or of the optimisation of the culture conditions discussed previously (see sections 5.2 - 5.4). Indeed, the responses reported here for unfractionated non-T cell stimulated AMLR are almost as high as those of non-adherent non-T cell stimulated AMLR responses, reported by Ng and Russell (1982). Similarly, the range of peak AMLR responses found

in the study of Sakane et al., (1978) were obtained using non-adherent non-T cell stimulated AMLR. All of the 38 normal subjects responded vigorously, having a range of 5,100  $\Delta$ cpm to 27,500  $\Delta$ cpm with a mean of 9,500  $\Delta$ cpm (Sakane et al., 1978).

Kalden et al., (1983) found wide variation in the AMLR of 56 normal controls using unfractionated non-T cells as the stimulating fraction. Although no values were stated for the range, graphical values were shown which ranged from approximately 3,000  $\Delta$ cpm to approximately 36,000  $\Delta$ cpm, having a mean of 10,830  $\Delta$ cpm  $\pm$  13,490. This wide variation is similar to that reported in this thesis.

Both Kalden et al., (1983), and Dock and Davey, (1980), found that approximately 20% of clinically healthy controls exhibit decreased AMLR. The results presented in this thesis suggested that approximately 25% of all clinically healthy individuals responded with a low magnitude. The results presented here suggested that control responses could be categorised, as low ( $<5,000$   $\Delta$ cpm), medium (5,000-10,000  $\Delta$ cpm) and high ( $>10,000$   $\Delta$ cpm). Although no previous studies have categorised the

observed responses as such, it is possible that the results obtained in other reports may be treated in a similar manner. Indeed, the study by Kalden et al., (1983), reports peak AMLR responses which could be placed into similar categories of low ( $<8,000 \Delta\text{cpm}$ ), medium ( $8,000-16,000 \Delta\text{cpm}$ ), and high ( $>16,000 \Delta\text{cpm}$ ) responses. Such classification may aid in the identification of real types of responsiveness in different individuals. These types of responsiveness may then correlate with genetic background or other factors which may affect the AMLR (Davey et al., 1984).

Despite the normal variation observed from day-to-day, the AMLR has been shown to be relatively reproducible with blood taken on consecutive days for normal individuals (Ng and Russell, 1982). However, the intra-individual variation in the AMLR over a much longer period of time has not yet been addressed. In the present study, the AMLR of ten clinically healthy individuals were studied at least three times and up to fourteen times over a period of three years. While several of the individuals' responses studied longitudinally were shown to be relatively reproducible, the responses of other individuals (6/10) showed wide variation on

repeat assay. These data, and the results of the AMLR for two individuals studied longitudinally over a period of three years, suggested that some form of seasonal variation in the AMLR response may account for the intra-individual variation. As discussed in the 'Results' section, a peak response appeared to occur in the summer months, although this may also be a reflection of the prevailing health conditions for each individual.

The variation in the normal AMLR must be established in order to compare normal AMLR and those found in autoimmune disorders. It has been shown that several physiological and genetic factors may affect the AMLR response even in normal individuals. Plasma cortisol (Ilfeld et al., 1977; Yu et al., 1978; Hahn et al., 1980; Indiveri et al., 1985), age and sex (Moody et al., 1981) and genetic (HLA-DR) phenotype (Davey et al., 1984) have all been implicated as contributory factors. Other endogenous factors, including hormones, may also affect the level of the AMLR, and these factors are subject to not only diurnal, but also seasonal or yearly cyclical variations. Further investigation of these parameters and their effects on the AMLR, may lead to a more complete understanding of not



only how the immune system becomes disrupted in autoimmune conditions, but also how, the immune system communicates with the nervous system and other tissues (see Besedofsky et al., 1983).

#### 5.6. Comparison of the AMLR in normal, myasthenic and rheumatoid subjects

The mean peak AMLR response of 22 myasthenic subjects with varying degrees of clinical symptoms has been shown in this report to be significantly decreased from the mean peak AMLR responses of 41 normal, clinically healthy individuals. A significantly decreased mean response was also shown for the AMLR responses of subjects with varying degrees of rheumatoid arthritis.

The AMLR has been shown to be depressed in individuals with SLE (Sakane et al., 1978; Kuntz et al., 1979), multiple sclerosis (Hafler et al., 1985) rheumatoid arthritis (Smith and Dehoratius, 1982), Hodgkins disease (Engleman et al., 1980) and Sjogren's Syndrome (Miyasaka et al., 1980), all diseases with an autoimmune aetiology.

The findings of this thesis are at variance with those of a previous report by Greenberg et al., (1984), that the AMLR was significantly increased in 11 of 15 pre-treatment MG patients. At least part of the apparent conflict may be accounted for by a difference in the classification of low and high responses between the two studies. In this thesis, low responses of the normal AMLR are classed as being less than 5,000  $\Delta$ cpm. High responses are classed as greater than 10,000  $\Delta$ cpm with the majority of normal responses falling into the medium range of 5,000  $\Delta$ cpm to 10,000  $\Delta$ cpm. In the study of Greenberg et al., (1984), of 15 patients studied pre-thymectomy, 8 patients had AMLR of less than 4,000  $\Delta$ cpm, 4 patients had net AMLR of between 5,000  $\Delta$ cpm and 10,000  $\Delta$ cpm, and 3 patients showed net AMLR greater than 10,000  $\Delta$ cpm. The classification of this group of myasthenics as 'hyperactive' with regard to the AMLR is equivocal. The AMLR of ten patients, followed post-thymectomy over a two year period, were suggested to have normalised. However, of these ten patients with 'normal' AMLR, eight patients exhibited net AMLR responses of less than 3,000  $\Delta$ cpm. One patient had an AMLR slightly less than 5,000  $\Delta$ cpm whilst the remaining patient had a net

AMLR response in the mid-range. Seven other MG patients who were not studied pre-thymectomy were also studied post-thymectomy. Of these seven post-thymectomy MG patients, only one responded in the AMLR in the 5,000  $\Delta$ cpm - 10,000  $\Delta$ cpm mid-range. All of the remaining six patients showed net AMLR responses of less than 3,000  $\Delta$ cpm. Thus, when conclusions are drawn from the data generated in a study, care must be taken to derive these conclusions from comparisons with established standards and controls. No such normal controls were presented in the study of Greenberg et al., (1984), resulting in the difficulties described above with regard to classification of response type.

As stated in the 'Results' section, the majority of the myasthenics studied in this thesis responded in the low ( $< 5,000 \Delta$ cpm) manner (14/22). All of these fourteen patients showed almost flat kinetic profiles for the AMLR over the nine day culture period. The seven myasthenics who responded in the mid-range, and the one myasthenic who responded with a high range normal-type response showed normal kinetic AMLR growth curve characteristics for medium responder, and high responder normal individuals respectively.

The limited study of the AMLR carried out on a group of seven patients with rheumatoid arthritis showed a depressed mean peak AMLR response in these patients in comparison to that of the normal group of individuals. Five of the seven R.A. patients responded with very low ( $<1,000 \Delta\text{cpm}$ ) AMLR, whilst the remaining two exhibited mid-range normal type responses. However, the kinetic profiles for the two mid-range R.A. responders were different to those observed for normal mid-range responders. A slow increase in the proliferative response for these two R.A. patients up to the peak response was followed by a rapid decrease in the response after the peak. This change in kinetics may be representative of an alteration in the cellular interactions that occur in the AMLR in disease states, and lends further support to the use of kinetic rather than single point studies of the AMLR.

The classification of the normal AMLR response into low, medium and high responder groups, and the observation of approximately 20% of the normal population responding in the low manner, may identify normal individuals who are predisposed to some form of immune dysfunction. Although the individuals who were identified as

responding in a low manner were all clinically healthy (Kalden et al., 1983; Richards ~~et al~~, 1986), the low AMLR response observed may serve as a predictor of future immune dysfunction by recognising individuals who are:

- i) already developing immune dysfunction but have no clinical symptoms of such internal disturbances,
- ii) predisposed by their low immune self-responsiveness to develop autoimmune or other diseases following sensitisation by an external or internal agent (eg virus or hormone).

Thus, despite the repeated observations of abnormal immunoregulation, as represented by the AMLR, in patients with diseases of the immune system, future efforts should be concentrated on the elucidation of the 'normal' AMLR and its significance.

#### 5.7. Conclusion: Immunoregulatory mechanisms in the AMLR

The significance of the AMLR in vivo, as well

as in vitro, has been questioned by several groups of workers who have suggested that the phenomenon is an artefactual response to xenogeneic antigens or to changes in cell populations during cell fractionation procedures (Huber et al., 1982; Kagen and Choi, 1983; MacDermott and Bragdon, 1983). However, the consistent and significant differences found between the AMLR of normal individuals and those of patients with various autoimmune diseases may argue against the 'artefactual AMLR' theory, proposed above. A major difficulty in the interpretation of results presented by different groups is the lack of standard culture conditions and separation protocols. Clearly, these operational differences could lead to some discrepancies between groups. However, the data presented in this thesis and those of many other studies on the AMLR in normal and disease states would suggest that the AMLR may play a significant role in the immunoregulation of the human immune system.

The major part of this thesis established the finding that the AMLR is defective in patients with myasthenia gravis, but no attempt was made to further dissect the AMLR to elucidate the nature of the defect. The theories and mechanisms which may account for the normal AMLR and how any defect

may arise will be discussed here.

The generation of helper activity by OKT4<sup>+</sup> cells and suppressor activity by OKT8<sup>+</sup> cells in the AMLR is established (Damle et al., 1981; Smolen et al., 1981). It has also been shown that OKT4<sup>+</sup> cells are necessary at the initiation of an AMLR (Damle et al., 1981; Smolen et al., 1981; Reinherz et al., 1982) and that OKT8<sup>+</sup> cells can only proliferate in the presence of interleukin-2 (IL-2) or autologous OKT4<sup>+</sup> cells (Smolen et al., 1982).

More recently, Kotani et al., (1984) have found that:

- i) T cell subsets capable of proliferating in AMLR mainly reside in OKT4<sup>+</sup>, but not OKT8<sup>+</sup> cells.
- ii) Cells recoverable from the AMLR express helper as well as suppressor activity.
- iii) OKT4<sup>+</sup> cells activated in the AMLR are able to mediate both the helper and suppressor functions.

- iv) Helper function mediated by  $\text{OKT4}^+$  cells is generated early in the course of an AMLR.
- v) Suppressor function in turn emerges from the activated  $\text{OKT4}^+$  population.

The observation that two distinct functions mediated by  $\text{OKT4}^+$  cells occur successively upon activation in AMLR, indicates that either there may exist both helper and suppressor subsets within  $\text{OKT4}^+$  cells, or that upon activation in AMLR, the same  $\text{OKT4}^+$  cells can become cells capable of expressing both helper and suppressor activities. Kotani et al., (1984) ruled out the latter possibility after finding that irradiation of  $\text{OKT4}^+$  cells that had been activated for 6 days in AMLR led to loss of all suppressor function but retention of substantial helper activity. However, their results indicated that a part of the AMLR may represent an important immune circuit among cells in the  $\text{OKT4}^+$  subset. These results may support those of Eardley et al., (1978) who suggested that a 'feedback suppression' mechanism may exist in the immune systems of mice.

The existence of some form of feedback-suppression mechanism within the immune system



would allow far greater flexibility for the responsiveness of the system. If the system was considered to be in constant, dynamic interaction then one may postulate that the response to a foreign antigen would be far quicker than if the system were in a resting state prior to antigenic challenge. Perturbation of the autologous interactions by the foreign antigen would result in a rapid amplification of specific helper subsets. In vitro, this would result in the generation of suppressor activity after approximately day 6 in culture (Kotani et al., 1984). However, in vivo, helper activity may continue to be stimulated whilst antigen persists in the body. Only after the majority of the antigen had been cleared would suppressor activity become apparent and feedback on the helper activity generated.

Alternatively, initial helper activity may stimulate suppressor activity after 6 days in vivo. However, due to the presence of stimulatory levels of antigen in the body, helper activity would overcome any suppressor activity generated and allow the clearance of the antigen. Following the clearance of the antigen, helper activity

would no longer be generated and suppressor activity would act on the effector functions generated during the initial helper phase. Fig 5.1 shows the possible interactions that may occur in the AMLR.

Whichever of these mechanisms may exist, if indeed they do exist, it is clear that some form of balance occurs between the level of help, suppression and antigen concentration. The term antigen concentration may be applied equally to foreign antigen or self-antigen (Ia). Thus, foreign antigen may tip the balance towards a conventional response to antigenic challenge, and self-antigen may, under the right conditions, upset the dynamic equilibrium established between help and suppression and result in the generation of an aggressive autoimmune state.

With regard to autoimmune states, very little data exists to support the above hypotheses. However, Sakane et al., (1983) demonstrated a completely defective AMLR activated OKT4<sup>+</sup> suppressor cell circuit in patients with SLE, a disease in which a defective AMLR has been found (Sakane et al., 1983, Takada et al., 1985). OKT4<sup>+</sup> cells were shown to be incapable of producing IL-2 and of expressing IL-2 receptors after activation

by autologous non-T cells. However, OKT8<sup>+</sup> cells from SLE patients were shown to be responsive to exogenous IL-2 (Takada et al., 1985). Defective non-T cell stimulatory capacity was ruled out by the addition of normal monocytes or exogenous IL-1 to SLE AMLR cultures, resulting in very little IL-2 production by SLE OKT4<sup>+</sup> cells.

Thus, if SLE OKT4<sup>+</sup> cells are effectively activated but fail to induce OKT4<sup>+</sup> suppressor cells, initial helper activity may continue to be stimulated. This may allow the uncontrolled expression of a pathological influence (autoantibodies, autoaggressive macrophages, natural killer cells, cytotoxic T cells).

It is possible that one or another aspect of the AMLR may be defective in different disease states. Thus, some patients may have excessive suppression of AMLR by macrophages, others may have a defect in the early stages of the AMLR, poor recruitment of cells, defective helper activity or suppressor function, or combinations of all these. Abberations in the levels of soluble factors (interleukins, interferons) may also play a role in different diseases.

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Studies of these details of the AMLR in

various disease states should shed light upon the cellular basis of immune abnormalities, and may provide a basis for a more effective investigation and ultimately, treatment.

## 6.0 EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS

### INTRODUCTION

The AMLR was suggested in Section One as representing a possible mechanism for immunoregulation in humans. This view has been supported by the increasing amount of data which have shown a decreased AMLR in diseases known to be associated with immunoregulatory dysfunction.

The aetiology of many autoimmune diseases is unclear and it is thought that most of these diseases have multi-factorial causes. Myasthenia gravis is unique as an autoimmune disease in the amount of data that have been generated on the autoantigen, the AChR. This has enabled the development of an animal model of the disease which bears similarities to the human condition.

The aim of the second part of this thesis is to study the AMLR in an animal model of MG, as the disease state is induced and possibly maintained. A study of the cellular immune responses, represented by the in vitro AMLR and mitogen responsiveness is intended to show the cellular changes that may occur during the establishment of EAMG, both in terms of anti-self immunoregulation (AMLR) and more general polyclonal responses (mitogen). Monitoring the humoral response,

represented by the serum anti-AChR antibody titre, is a measure of B cell activity in the immune system, and thus of the degree of immunoregulation.

### 6.1 History of EAMG

Experiments carried out in 1973 by Patrick and Lindstrom, and Patrick et al, yielded early experimental evidence that an autoimmune response to AChR was the pathogenic mechanism occurring in MG. Experimental autoimmune myasthenia gravis (EAMG), discovered in these experiments, has been comprehensively reviewed by Lindstrom (1979), Vincent (1980), and Harrison and Behan (1986) and the model in alternative animals will be discussed here.

### 6.2 Rabbits

The induction of EAMG in rabbits immunised with AChR purified from electric eel or electric ray has been demonstrated by many workers (Sugiyama et al, 1973; Heilbronn and Mattson, 1974; Aharonov et al, 1975; Green et al, 1976; Heilbronn et al, 1976; Penn et al, 1976; Sanders et al, 1976; Valderama et al, 1976; Fumagalli and Clementi, 1978; Takamori et al, 1979; Ueno et al, 1980; Niemi et al, 1982) following the initial observations of Patrick and Lindstrom (1973).

EAMG generally becomes apparent about 1 month after immunisation with 100-400 $\mu$ g of purified AChR in complete Freund's adjuvant (CFA), or a few days after a booster injection given 2-4 weeks after the initial immunisation. High serum anti-AChR antibody titres are obtained by using frequent booster injections (Lindstrom et al, 1981; Elfman, 1984).

The clinical symptoms of EAMG include a flaccid paralysis which affects the head, neck and back muscles. Affected animals show drooping head and ears, have difficulty in sitting and often lie flat with extended limbs. Severe cases can exhibit wheezing and loss of appetite. Rabbits with EAMG commonly die 1-2 days after the onset of symptoms, often as a result of respiratory failure with associated pneumonia (Elfman, 1984). Muscular weakness is shown by decremental electromyographic responses to repetitive nerve stimulation and is temporarily alleviated by anti-cholinesterase therapy.

EAMG has also been induced in rabbits by using AChR purified from denervated rat muscle (Wonnacott et al, 1982), foetal calf muscle (Lindstrom, 1979), chick muscle (Sumikawa et al, 1982; Dolly et al, 1983), cat muscle (Dolly et al, 1983) and a mouse muscle cell line (Boulter and Patrick, 1977).

Immunisation of rabbits with SDS-denatured AChR or its denatured subunits (Lindstrom et al, 1978) or with reduced and carboxymethylated whole Torpedo AChR (Bartfeld and Fuchs, 1977) did not result in clinical signs of EAMG. However, Fuchs et al, (1981) showed the induction of EAMG in rabbits immunised with a tryptic fragment ( $M_R$  27,000) of Torpedo AChR.

### 6.3 Rats

In general, rats are slower to develop clinical symptoms of EAMG than rabbits (Vincent, 1980), although different strains of rats show varying degrees of susceptibility to disease induction (Green et al, 1975; Vincent, 1980). Lewis and Lou rats being most susceptible in this respect (Lennon et al, 1978; Lindstrom et al, 1981).

A single immunisation of 1-100 $\mu$ g of purified Torpedo AChR in CFA at multiple intradermal sites, was shown to produce chronic EAMG, in Lewis rats, although frequent booster injections were required to produce high antibody titres (Lennon et al, 1975; Lindstrom et al, 1981).



Resistance to EAMG has, however, been reported in Lewis rats immunised with Torpedo AChR in CFA (Sanders et al, 1976; Kim and Sanders, 1981). Lou rats immunised with purified bovine and human AChR in CFA (Einarson et al, 1982) show EAMG and Wistar rats have shown weakness when immunised with Narke (an electric ray) receptor in the absence of CFA (Ueno et al; 1982). However Wistar rats have failed to show clinical weakness when immunised with Torpedo AChR, despite evidence of serum anti-AChR antibodies and reduced m.e.p.p. amplitudes (Green et al; 1975; Alema et al, 1981).

Mild EAMG has been induced in Lewis rats by immunisation with individual subunits of Torpedo AChR. Using high doses (50 $\mu$ g) and frequent boosters (4-5 over 7-10 weeks), Lindstrom et al., (1978, 1979) were able to show that all of the subunits were immunogenic although the  $\alpha$  and  $\delta$  subunits proved to be the most effective. This is in contrast to the efforts of the same and other groups (Lindstrom et al, 1978; Mehraban et al, 1982; Dolly et al, 1983) who were unable to induce EAMG by immunisation of rabbits with AChR subunits. Claudio and Raftery (1977, 1980 a, b) also failed to induce clinical EAMG in rats or rabbits by similar means.

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Clinical EAMG in rats immunised with eel AChR in CFA becomes apparent about 1 month after the initial injection (Lennon et al, 1975). Weakness persists until the animal dies but it may survive and remain in this condition for more than 80 days (Lindstrom, 1977). The clinical features of chronic EAMG in rats closely resemble those of MG in humans.

Lewis rats immunised with eel AChR, together with both B.pertussis and CFA, develop a transient acute phase of muscular weakness between 8 and 11 days after the injection. Although this stage is characterised by massive phagocytic infiltration of the muscle endplates (Engel et al, 1976 a,b), weakness lasts only for a few days before the animal regains clinical normality. This apparent normality is maintained until the onset of the chronic phase of EAMG, 30 days after immunisation. It is not known whether the acute phase is an artifact of the use of pertussis, or whether the additional adjuvant enhances cellular mechanisms that normally occur in EAMG. It is of interest that acute EAMG can be induced in the absence of pertussis in rabbits injected with Torpedo AChR (Fumagalli and Clementi, 1978) and in mice injected with denatured rat muscle AChR (Granato et al, 1980).

#### 6.4 Mice

The involvement of genotype in the susceptibility of animals to EAMG is highlighted by the use of the various inbred, congenic and recombinant strains of mice that are available. Fuchs et al., (1976) demonstrated that 10 (out of 14) strains of mice that developed EAMG possessed haplotypes H-2<sup>a</sup>, H-2<sup>b</sup>, H-2<sup>d</sup> and H-2<sup>k</sup> of the major histocompatibility complex (MHC). Haplotypes H-2<sup>q</sup> and H-2<sup>s</sup> were shown to be present in resistant strains (Fuchs et al, 1976). This strain dependence of clinical weakness was later confirmed by Berman and Patrick, (1980 a,b; Berman et al, 1981) who also showed that specific haplotypes, particularly H-2<sup>b</sup>, Iq-1<sup>b</sup> accounted for the incidence of paralysis.

An additional experimental advantage that mice offer as an animal model of EAMG is their well characterised and manipulatable immune system. In early studies of congenic mice by Christadoss et al, (1981), clinical muscle weakness and mean m.e.p.p. amplitude reduction correlated well with the mean cellular immune responses to Torpedo AChR mice. De Baets et al, (1982), showed that the magnitude of the in vitro cellular response to Torpedo AChR was directly proportional to the total amount of serum anti-AChR antibody and severity of disease as measured by muscle AChR content. However, in a later study, Christadoss and colleagues failed to demonstrate a correlation

between AChR-specific lymphocyte responses and muscle AChR loss when individual B6 mice were tested (Christadoss et al, 1985).

Anti-AChR responses, both cellular and humoral, and susceptibility to EAMG were found to be I-A region (MHC) controlled (Christadoss et al, 1981). Anti-(Ia antigen) antiserum can eliminate or partially suppress proliferative responses against the AChR (Christadoss et al, 1981, 1982, 1983a; Waldor et al, 1983). More detailed discussion of murine cellular responses in EAMG will follow later. The review of Harrison and Behan (1986) concluded that the variation in strain susceptibility may depend upon differences in the specific anti-AChR immune response, rather than on differences in neuromuscular transmission sensitivity.

#### 6.5 Mode of action of anti-AChR antibodies

Impaired neuromuscular transmission in EAMG, and indeed in MG, has been considered by many workers to result from a decrease in the number of active receptors at the muscle endplate. Experiments in which rats have been injected with immunoglobulin from 'chronic-phase EAMG' rats i.e. passive transfer, have indicated that anti-AChR antibodies play a major role in the decrease in the number of functional receptors (Toyka et al., 1975, 1977, 1978, 1980).

Although it is generally assumed that anti-AChR antibodies play an important part in the pathogenesis of EAMG, inconsistent results have been obtained in correlating the impairment of neuromuscular transmission with anti-AChR antibody titre. The vast excess of antibody over receptor that may exist in the chronic phase (1000:1) (Lindstrom et al, 1976 a) appears to support the existence of particularly important sub-populations of antibodies. However, the recent report of Bionda et al, (1984) showed no difference between the isoelectric focussed patterns of serum anti-AChR antibodies and those eluted from the muscle of rats with EAMG.

Antibodies of particular importance in the disease process have been suggested as those that are directed against the acetylcholine binding site, so-called anti-'site' antibodies. Anti-site antibodies are normally assayed by the level of blocking of

$\alpha$ -bungarotoxin to AChR. However, several groups have shown the presence of anti-'site' antibodies by using a modified radioimmunoassay which does not employ radiolabelled  $\alpha$ -bungarotoxin (which blocks the acetylcholine binding site). (Sugiyama et al, 1973; Lindstrom et al, 1976; Trotter et al, 1977; Alema et al, 1978). Binding of antibodies to sites around the

acetylcholine binding site could result in blocking access of the acetylcholine to the binding site (Barkas et al, 1982).

The existence of a single myasthenogenic determinant is made less likely by the observations (Lindstrom et al, 1978, 1979; Elfman et al, 1983) that all four subunits (  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  ) of Torpedo AChR are capable of inducing EAMG, despite observations to the contrary in a later study by Claudio and Raftery (1980b). However, various groups of workers have described the induction of EAMG by the passive transfer of monoclonal antibodies directed at different 'non-site' AChR antigens. (Richman et al, 1980; Burres et al, 1981, Gomez et al, 1981, Lennon and Lambert, 1981; Kamo et al, 1982; Gomez and Richman, 1985).

A phylogenetically conserved main immunogenic region (MIR) on the  $\alpha$ -subunit of Torpedo, eel, foetal calf and human AChRs has been described by Lindstrom and colleagues (Tzartos and Lindstrom, 1980; Tzartos et al, 1981, 1983, 1985; Lindstrom et al, 1981, Lindstrom, 1982, 1984) which is distinct from the acetylcholine and toxin binding site. The majority of anti-AChR antibodies (approximately 60-70%) in experimental rats (Tzartos and Lindstrom, 1980; Tzartos et al, 1981, 1983) and in MG patients (Tzartos et al, 1982) are directed to this region.

While the antibody binding site(s) on the AChR is gradually being determined, the way in which the antibody reduces the functional ability of the receptor remains unclear. The studies carried out so far have been comprehensively reviewed by Harrison and Behan (1986).

As for MG itself, the mechanisms by which these anti-AChR antibodies act have been suggested to be:

- i) Direct block of AChR function.
- ii) Increased degradation or decreased synthesis of the AChR, or both.
- iii) Complement-mediated lysis

or any combination of these possible mechanisms.

i) Direct Block of AChR function

The pathogenic activity of anti-AChR antibodies may reside in their ability to reduce or inhibit the electrophysiological response, or their inhibition of the binding of cholinergic ligands. The electrophysiological response to carbamoylcholine after pre-incubation of isolated eel electroplaques with rabbit (Patrick et al, 1973; Sugiyama et al, 1973; Penn et al, 1976; Karlin et al, 1978), or rat and goat

(Lindstrom et al, 1976, 1977) anti-eel AChR antisera has been found to be inhibited. Complement is not involved (Lindstrom et al, 1977) and toxin binding activity is only slightly impaired (Lindstrom et al, 1977; Karlin et al, 1978).

Various reports have suggested that inhibition of the electrophysiological response may be due to a freezing of the ion channel in a closed conformation (Hess et al., 1975; Karlin et al., 1978; Desouki et al., 1981). Inactivation of the ion-channel may also be due to uncoupling of the active site and the ion-channel by binding of antibody to a site distinct from the acetylcholine or toxin binding sites. (Eldefrawi et al., 1978; Eldefrawi and Eldefrawi, 1980).

Anti AChR antibody inhibition of the binding of  $\alpha$ -toxin or cholinergic ligand to membrane bound receptor has yielded variable results (Lindstrom et al, 1976, 1977; Sanders et al, 1976; Eldefrawi et al, 1979; Martinez-Carrion et al, 1981; Farach et al, 1982; Desouki et al, 1983), although findings with solubilised eel or Torpedo AChR have been more consistent. Autologous antibodies (Penn et al, 1976; Aharonov et al, 1977; Zurn and Fulpius, 1977; Karlin et al, 1978; Claudio and Raftery, 1980a), or F(ab')<sub>2</sub> fragments (Wonnacott et al, 1980) have yielded 50-100%



inhibition of  $\alpha$ -toxin binding to detergent solubilised AChR, similar results being found for the inhibition of acetylcholine binding (Sanders et al, 1976).

Inhibition of  $\alpha$ -toxin binding to soluble or membrane bound Torpedo receptor has been shown by using Fab fragments (Martinez-Carrion et al, 1981; Farach et al, 1982) and by using whole antisera or Fab fragments (Claudio and Raftery, 1980a). It was proposed that antigenic modulation of the AChR was unlikely to account for the observed block of AChR function following these observations (Claudio and Raftery, 1980a).

Anti-'site' antibodies have been suggested to be physiologically important in the pathogenesis of EAMG (Zurn and Fulpius, 1977; Claudio and Raftery, 1980 a,b) despite observations in passive transfer experiments to the contrary (Lennon and Lambert, 1980, 1981; Richman et al, 1980; Gomez et al, 1981; Kamo et al, 1982).

Evidence exists both for the inhibition of agonist-mediated responses without affecting the acetylcholine binding site and also for the direct blockade of binding of  $\alpha$ -toxin or cholinergic ligands to their binding sites. Whether the pathogenic effect is produced by antibodies directed at the acetylcholine binding site, at an adjacent site, or both, remains unclear.

ii) Receptor turnover

The dynamic replacement of receptor in the membrane has been followed by using radiolabelled  $\alpha$ -bungarotoxin and measuring the release of radioactivity into the extracellular fluid (Devreotes and Fambrough, 1975). Studies with normal muscle cells have shown that the half-lives of both junctional and extra-junctional receptors are reduced in the presence of anti-receptor antisera (Heinemann et al, 1978; Reiness et al, 1978). A two-fold increase in the rate of degradation of both junctional and extra-junctional receptor has also been observed in the diaphragm tissue of rats with chronic EAMG in comparison to normal controls (Merlie et al, 1979).

Increased degradation of the receptor in cultured rat muscle cells was earlier shown to be caused by IgG purified from rat anti-eel AChR antiserum from animals with EAMG (Heinemann et al, 1977). This was prevented at 22°C or by the presence of metabolic inhibitors. Accelerated degradation of AChR on cultured muscle cells was induced by anti-AChR IgG (Lindstrom and Einarson, 1979) and (Fab)<sub>2</sub> fragments, Privès et al, (1979) but not by Fab fragments (Lindstrom and Einarson, 1979).

Fumagalli and co-workers (Engel and Fumagalli, 1982; Fumagalli et al, 1982) found that increased receptor degradation may be accompanied by a relative decrease in receptor synthesis 'in vivo', and suggested that a disorganised cytoskeleton or post-synaptic membrane may prevent the 'normal' rate of replacement.

### iii) Complement

The role of complement in EAMG was indicated by Toyka et al (1977) and Howard and Sanders (1980) who showed that C-3 depleted mice and rats were less sensitive to the effects of the passive transfer of EAMG by human myasthenic sera. However, mice genetically deficient in C-5 were shown to react similarly to normal controls (Toyka et al, 1977) suggesting that lysis initiated by activation of C-5 was not involved in the pathogenesis of EAMG. The role of complement in EAMG is however, equivocal. Ultra structural studies by Engel and colleagues showed the presence of IgG and C-3 on the junctional folds of Lewis rats injected with immunoglobulin from rats with EAMG and in EAMG rats themselves (Sahashi et al, 1978; Engel, 1979). However, Lennon (1978) was unable to show that serum from rats with EAMG could lyse cultured foetal rat muscle cells in the presence of complement.

Which mechanism, or combination of mechanisms is involved in the pathogenesis of EAMG remains unsolved. It would seem very likely that, in a disease caused by many factors, the pathogenesis would occur as a result of various mechanisms.

#### 6.6 Cellular reactivity in EAMG

One of the distinctions between the 'acute' and 'chronic' phases of EAMG in rats is the presence of inflammatory cells or macrophages that infiltrate the muscle endplates in the acute stage (Engel et al, 1976). Why phagocytic infiltration presumably caused by the IgG and C-3 bound to the membrane, is seen in the acute phase, and not in the chronic phase (in which IgG and C-3 are also present) is not known. Massive phagocytic invasion of the synaptic cleft is also observed in passive EAMG, and closely resembles that seen in acute EAMG (Engel et al, 1978). This indicates that the cellular invasion in acute EAMG is not produced by anti-AChR cells, but by non-specific cells responding to bound antibody and complement. During the acute phase only, delayed-type hypersensitivity to intradermal injection of AChR occurs (Lennon et al, 1976). The importance of this hypersensitivity to the cellular events occurring at the endplates, is not known. Rats depleted of C-3 prior to injection with anti-AChR antibodies from chronic EAMG rats, or with purified AChR, do not show clinical signs of EAMG, nor

do they show phagocytic invasion of the muscle endplates (Lennon et al, 1978).

A specific role for macrophages in the on-going pathogenesis of EAMG cannot readily be envisaged.

Lennon et al, (1976) showed, by cell depletion experiments in rats, that the response to AChR was T-cell dependent. Thymectomy and whole body X-irradiation followed by reconstitution with non-T cells only, prevented the development of anti-AChR antibodies or signs of EAMG (Lennon et al, 1976). These observations were supported by the report of Tarrab-Hazdai et al, (1975) who showed that the transfer of lymph node cells from affected animals could induce EAMG. Induction of EAMG by this adoptive transfer was shown to be suppressed by the removal of T cells from the donor cells (Fuchs et al, 1978).

Lymphocytes isolated from rats immunised with Torpedo AChR proliferate in response to both purified Torpedo AChR and unpurified rat muscle AChR (Noguchi et al, 1980). Further studies using murine lymph node cells showed that AChR-induced proliferation was blocked by alloantisera that identified a  $\text{Lyt-1}^+, 23^-$  helper T cell (Christadoss et al, 1981). Stimulation of the T cells was shown to be dependent on macrophages, a later study indicating that macrophages presented the AChR to specific T cells in association with Ia antigen on the surface of the macrophage (Christadoss et al, 1982).

AChR-activated blast cells (Hohlfeld et al, 1981a,b; Wekerle et al, 1981) and AChR-activated murine lymphocytes (Christadoss et al, 1981; Bogen et al, 1984) have been identified in different assay systems as helper T cells. The study of DeBaets et al, (1982) identified the proliferative cell in the in vitro anti-AChR response as a helper T cell, both phenotypically and functionally. Reasonable correlation was observed between the in vivo anti-AChR antibody responses, in vitro anti-AChR production and the loss of muscle AChR. Both T helper cells and macrophages were found to be necessary for the observed effects (DeBaets et al, 1982). Defective macrophage activity in the adherent cell population of mice with EAMG was suggested as a possible cause of the reduced T-cell mitogen responses of the affected animals (Christadoss et al, 1983b). Excessive suppression of the Con-A induced T cell response was observed by AChR-sensitised macrophages, whereas AChR-sensitised T cells responded normally to Con-A in the presence of non-sensitised, syngeneic macrophages (Christadoss et al, 1983b). The cause of the expression of this macrophage suppression is not known, although macrophage suppression has been observed in mixed lymphocyte responses (Weiss and Fitch, 1978), AMLR (Smolen et al, 1981) and mitogen induced lymphoproliferation (Raff and Hinrichs, 1977).

Pachner and Kantor (1984) and Bogen et al, (1984) have both described AChR-specific suppressor T cells in mice immunised with Torpedo AChR. Both groups underlined the possible importance of the specific suppression of the anti-AChR response in humans.

#### 6.7 Choice of the rabbit model

The present study of the AMLR in EAMG was initiated using New Zealand white rabbits because these animals are susceptible to the disease (Patrick and Lindstrom, 1973) and provide a relatively good source of PBL which may be separated (Wilson et al, (1975b). Repeated determinations on PBL from the same animal allow the longitudinal study of various parameters (Sheppard et al, 1977). This is not possible with the low blood volumes of mice or rats, and the use of other organs as sources of T and non-T cells was considered to be less relevant.

12 six week old animals were immunised with AChR purified from Torpedo marmorata and levels of circulating anti-AChR antibodies were measured by R.I.A. Changes in the cellular immune responses of the rabbits were also studied during the induction and maintenance of the disease state. Although the humoral immune responses to heterologous AChR have been well documented (reviewed in Harrison and Behan, 1986), changes in immune cellular properties following the induction of EAMG have not been so extensively studied.

The alterations in cellular responses in rabbits with EAMG are the subject of this section.

Although the immune systems of mice and man have been investigated in detail, much less is known about the participating cells in rabbit immune responses. The advantages and disadvantages of studying the rabbit immune responses are summarised below:-

#### Advantages

- i) Responses of rabbit PBL to PHA and Con-A are well-documented. (Teodorescu et al, 1976; Ozer and Waksman, 1974; Shek et al, 1974).
- ii) An AMLR has been demonstrated between mesenteric lymph node cells (responders) and autologous spleen cells (stimulators) (Watkins et al, 1984).
- iii) Rabbit one-way MLR has been consistently demonstrated with PBL, and AMLR may be induced in PBL under certain circumstances (Milthorp and Richter, 1979).
- iv) Rabbit T cells have been phenotypically identified by anti-rabbit T cell monoclonal antibodies (McNicholas et al, 1981; Watkins et al, 1984).
- v) Mouse anti-rabbit T cell ascites fluid (R.I.A., U.K. Limited) is commercially available



- vi) PBL can be repeatedly isolated from the same animal in sufficient quantities, allowing longitudinal studies to be performed.

#### Disadvantages

- i) Very little experimental data exist for rabbit AMLR.
- ii) Difficulties in obtaining mixed lymphocyte reactions with rabbit PBL have been reported (Harrison et al, 1971; Knight et al, 1971; Ling and Kay, 1975; Sheppard et al, 1977; Watkins et al, 1984), although improved culture conditions may overcome this potential problem.
- iii) Inbred strains are not available.

## 7.0 METHODS

### 7.1 Iodination of $\alpha$ -bungarotoxin to high specific activity

Iodination of  $\alpha$ -bungarotoxin was carried out according to the method of Hunter and Greenwood (1962). 0.05M potassium phosphate, pH 7.5, (reaction buffer, 250ml), and 0.01M potassium phosphate, pH 7.5 (elution buffer, 100ml) containing 1% (w/v) bovine serum albumin were prepared as stock solutions and stored at 4°C. Immediately prior to use, solutions of chloramine T (5mg/ml, 10ml), sodium metabisulphite (0.16 mg/ml, 10ml) and potassium iodide (10mg/ml, 10ml) were separately prepared in reaction buffer.

Sephadex-G25 (4g) was allowed to swell in reaction buffer (30ml) for 30 min and packed in a glass column (1cm x 30cm). The packed column was washed with elution buffer (30ml) and drained until the top of the column was just covered with buffer.  $\alpha$ -bungarotoxin (2.5 nmol) in 0.1M sodium phosphate buffer, pH 7.5 20 $\mu$ l was added to the reaction tube together with Na <sup>125</sup>I (20 $\mu$ l, 2mCi). The reaction was started by adding chloramine T (10-1) and, after stirring for 60 sec at room temperature, was stopped by the addition of sodium metabisulphite (0.75ml) and potassium iodide (0.2ml). The reaction mixture was then transferred by glass pasteur pipette to the top of the column and allowed to drain in. The iodinated toxin was eluted from the column with elution buffer (20ml) and fractions (1ml)

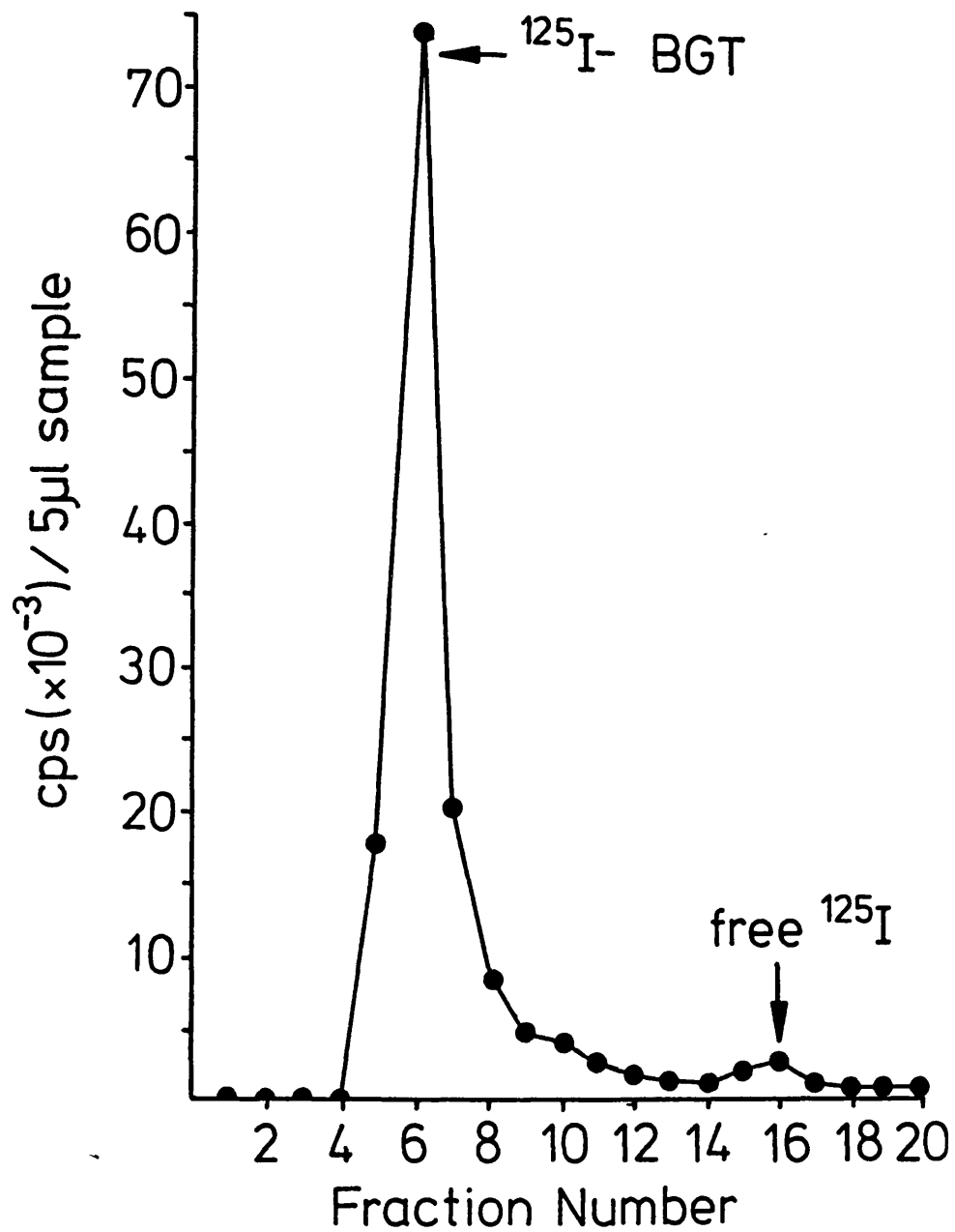


Figure 7.1 The elution profile of iodinated  $\alpha$ -bungarotoxin and free  $^{125}\text{I}$  from a Sephadex G-25 column.

of the column eluate were collected. Aliquots (5 $\mu$ l) from each fraction were placed in stoppered LP2 tubes and counted for gamma emissions in an LKB 1280 Ultrogamma counter. Fractions corresponding to the major peak (Fig. 7.1) were collected.

A final specific activity of the pooled samples was typically found to be 760 Ci/mmol which represents approximately 80% incorporation of the radiolabel into the  $\alpha$ -toxin.

## 7.2 Preparation of the anti-AChR affinity column

Affinity columns used for the purification of AChR are most commonly prepared by coupling  $\alpha$ -toxin to activated agarose through some of the many lysine residues present on the toxin. Naja naja siamensis toxin is used because it is available in large quantities and its binding to AChR is more reversible than that of  $\alpha$ -bungarotoxin. The method of Lindstrom et al, (1981) was followed.

Sepharose 4B (250g wet beads) was washed with water (1-2L) under reduced pressure, and the beads were resuspended in water (500ml) at 10°C. The pH was adjusted to 11.0 with 2M NaOH and cyanogen bromide (25g) was added to the stirred solution, care being taken to maintain the pH at 11.0, (by addition of NaOH) and the temperature at approximately 10°C. After all the cyanogen bromide had dissolved, the suspension

was applied to a Buchner funnel. The beads were washed with ice-cold water (1L) followed by 0.2M sodium carbonate buffer pH 9.5, (2L) at 4°C. The beads were not allowed to dry out at any time. They were then resuspended in 0.2M sodium carbonate buffer, pH 9.5 (500ml) at 4°C and, while the suspension was gently agitated, the  $\alpha$ -toxin (10mg/ml, in 0.1M sodium phosphate, pH 7.5, 12.5ml) was added dropwise. The suspension was agitated gently at 4°C overnight, after which the absorbance (280nm) of the supernatant changed from an initial value of 0.265 to a final, complete conjugation value of 0. Glycine (1M, 500ml) was added and the suspension was left at room temperature overnight with gentle agitation. The suspension was then poured into a chromatography column (3 cm x 20 cm), washed with 10mM potassium phosphate buffer containing Triton X-100 (2% v/v), 1mM EDTA and 0.02% w/v  $\text{NaN}_3$  at pH 7.4 and stored at 4°C until use.

### 7.3 Preparation of AChR from *Torpedo marmorata*

Acetylcholine receptor was purified from the electric organs of the electric ray *Torpedo marmorata* by a modified method of Wonnacott et al, (1980).

Frozen electric organ (200-300g) was homogenised in 0.4M NaCl (300ml) containing 10mM potassium phosphate, 1mM EDTA, and 0.02% (w/v)  $\text{NaN}_3$ , pH 7.4 (buffer A) using a Sorvall Omnimixer. The homogenised tissue was centrifuged at 100,000g for 30 min in a

Beckman L5-50B Ultracentrifuge (S35 rotor). The pellets were resuspended in 10mM potassium phosphate (300ml) containing 2% (v/v) Triton X-100, 1mM EDTA, and 0.02% (w/v)  $\text{NaN}_3$ , pH 7.4 (buffer B), stirred overnight at 4°C and then centrifuged at 100,000g for 45 min (Beckman L5-50B, S35 rotor). The supernatant was stirred with Naja naja siamensis  $\alpha$ -toxin coupled covalently to Sepharose 4B (50ml packed beads) for 4 h at room temperature. The beads were then washed alternately with 200mM NaCl (1L) and 1M NaCl (1L) both containing 50mM potassium phosphate, 0.1% (v/v) Triton X-100, 1mM EDTA, and 0.02% (w/v)  $\text{NaN}_3$ , pH 7.4. The beads were packed into a chromatography column (3 cm x 30 cm) and the AChR was eluted with 10 mM benzoquinonium chloride, containing 10mM potassium phosphate and 0.1% (v/v) Triton X-100, pH 7.4. The eluted AChR was passed directly onto a equilibrated DE52 ion-exchange column (3 cm x 10 cm) and the eluate from this column was recycled onto the toxin-bead column. The eluates from both columns were recirculated in this way overnight at 4°C by using a Gilson peristaltic pump. The DE52 column was washed with 10mM potassium phosphate containing 0.1% Triton X-100, pH 7.4 and AChR was eluted from this column with 1M NaCl (100ml) containing 10mM potassium phosphate, 0.1% Triton X-100, pH 7.4. The receptor preparation was assayed for protein content and  $\alpha$ -bungarotoxin-binding activity and extensively dialysed against PBS, pH 7.2 before use in any immunisation procedures. The amounts of receptor

present in the crude extract and in the supernatant from the toxin beads (after incubation) were determined by using the ammonium sulphate assay (Meunier et al, 1972). The amount present in the final preparation was determined by using the toxin binding assay described by Schmidt and Raftery, (1973). A summary of the purification for AChR may be seen in Figure 7.2.

THE PURIFICATION OF AChR FROM THE ELECTRIC ORGAN TISSUE  
OF TORPEDO MARMORATA

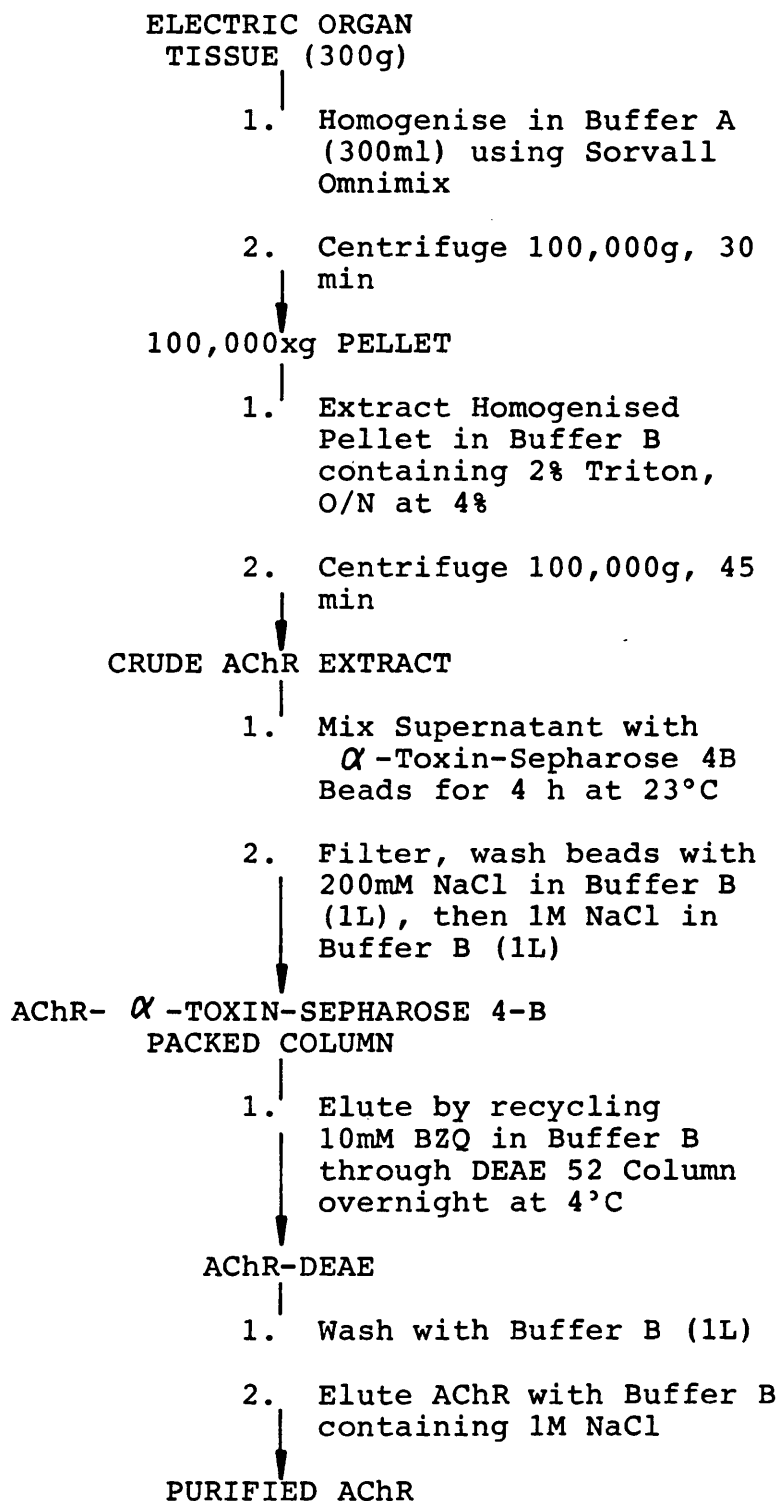


Figure 7.2



7.4 Determination of toxin binding activity in the crude AChR preparation by the ammonium sulphate precipitation assay

For the assay of crude receptor preparations, an ammonium sulphate precipitation assay has been developed from the method of Meunier et al, (1972). A crude receptor sample (100 $\mu$ l) was mixed with  $^{125}\text{I}$ -

$\alpha$ -bungarotoxin ( $\alpha$ -BGT) (1.5 nM, 50 $\mu$ l) and incubated for 45 min at room temperature. Saturated ammonium sulphate (133  $\mu$ l) was added to yield a final concentration (v/v) of 40%. The samples were left for 16 h at 4°C, before addition of 40% (v/v) ammonium sulphate solution (1ml) and the samples were filtered on Whatman GF/C filter discs. Each filter disc was washed with 40% (v/v) ammonium sulphate solution (3ml) and counted in an LKB 1280 Ultrogamma counter. The specific binding was measured by carrying out all assays in the presence and absence of 0.1M benzoquinonium chloride (50 $\mu$ l), and the  $^{125}\text{I}$ - $\alpha$ -BGT that remained bound in the presence of this competing ligand was defined as non-specifically bound. Receptor activity was expressed as the molarity of  $^{125}\text{I}$ - $\alpha$ -BGT binding sites.

### 7.5 DEAE - Cellulose Filtration Assay

Assay of purified acetylcholine receptor was performed by filtration on DEAE-cellulose discs, essentially as described by Schmidt and Raftery, (1973).

Purified receptor (100 $\mu$ l) was incubated with  $^{125}\text{I}$   $\alpha$ -BGT (1.5nM, 50 $\mu$ l) for 90 min at room temperature, in the presence and absence of 0.01M benzoquinonium chloride (50 $\mu$ l). The reaction was terminated by the addition of 10mM potassium phosphate (1ml, pH 7.4), containing 1% (v/v) Triton X-100, and 0.1% (w/v) bovine serum albumin. The sample was filtered through two DE81 cellulose filter discs (2cm diameter) and the discs were washed with the above buffer (3ml). The discs were then counted in an LKB 1280 Ultrogamma counter and the specific activity calculated and expressed as above.

### 7.6 Preparation of the AChR for immunisation

The AChR prepared by affinity chromatography (see Results, p198 for specific activity) was extensively dialysed against several changes of PBS, pH 7.2, (5L) in Visking tubing at 4°C over 3 days. The dialysed AChR preparation was then filter-sterilised through a 0.2 $\mu$ m sterile filter unit and stored at 4°C in sterile bijou bottles until used. A water-in-oil emulsion of the AChR was prepared by forcing the AChR preparation

(1 vol) at the required concentration into Complete Freund's adjuvant (3 vol) by using a double hubbed glass syringe apparatus. An adequate state of emulsification was judged by the inability of a drop of the emulsion to disperse when placed on water. In all subsequent secondary immunisation procedures, the AChR protein in PBS was emulsified in Incomplete Freund's adjuvant (IFA).

#### 7.7 Immunisation with the AChR

In an attempt to avoid a sudden, acute phase of EAMG in the rabbits, repeated low doses of AChR in adjuvant were given. Thus, AChR protein (80 $\mu$ g) in PBS emulsified in CFA (1ml) was injected intra-muscularly, (0.5ml) in each hind limb, by a trained animal technician.

Subsequent secondary immunisations consisted of a similar protocol, except that AChR 50 $\mu$ g in IFA was injected, split between the two hind limb sites. Each AChR preparation had a specific activity of approximately 5pmol/ $\mu$ g.

All control rabbits were injected with an emulsion (1ml) of PBS in CFA or IFA (1 vol : 3 vol), split between the two hind limb sites.

## 7.8 Radioimmunoassay for rabbit anti-(AChR) antibodies

The radioimmunoassay for the detection of rabbit anti-(AChR) antibodies was a modification of the method of Lindstrom (1977).

Solubilised AChR from Torpedo marmorata (0.05nM) in PBS was incubated with  $^{125}\text{I}$ - $\alpha$ -BGT (0.5nM) in PBS for 45 min at room temperature. Test serum (5 $\mu$ l) was added, diluted when necessary with normal rabbit serum, and the mixture was incubated overnight at 4°C, or for 2h at room temperature. Goat anti-(rabbit IgG) antiserum (25-30 $\mu$ l) was added and a precipitate was allowed to form overnight at 4°C or for 2 h at room temperature. The precipitate was separated by centrifugation at 3000g for 10 min, the pellet was washed twice with 0.01M NaCl containing 1% Triton X-100, and counted in an LKB 1280 Ultrogamma counter. Control samples were pre-incubated with 25mM benzoquinonium chloride before addition of  $^{125}\text{I}$ -

$\alpha$ -BGT; the counts that remained after addition of this competing ligand were defined as non-specifically bound. Titres were expressed as moles  $^{125}\text{I}$ - $\alpha$ -BGT binding sites precipitated per litre of serum.

### 7.9 Isolation of rabbit peripheral blood leucocytes

Rabbit blood (20ml) was taken by cardiac puncture from an anaesthetised New Zealand white rabbit and collected in a sterile heparinised (400U) universal tube. The blood was mixed with sterile 3.5% (w/v) dextran in PBS (pH 7.2) and incubated at 37°C for 30 min, occasionally tapping the sides of the tube to dislodge any erythrocytes. The leucocyte-rich supernatant (8ml) was layered carefully onto a Ficoll-Hypaque density gradient (3ml) and centrifuged at 400g for 30 min. The band of leucocytes at the interface was harvested by using a sterile, glass Pasteur pipette and washed twice with RPMI 1640 before counting the cells in a haemocytometer counting chamber as described (Methods, p66). The cell suspension was then resuspended to  $3 \times 10^6$  cells/ml in RPMI 1640.

### 7.10 Preparation of antibody-coated ox red blood cells

Antibody-coated ox red blood cells (ORBC) were prepared according to the method of Ling et al, (1977), with slight modifications. ORBC in Alsevers' solution were washed five times in PBS by centrifugation at 200g for 10 min, taking care to remove the buffy coat cells after each wash. The packed erythrocytes were resuspended to 10% (v/v) in RPMI 1640. Goat anti-mouse IgG (3ml, 1mg/ml) was added to the washed ORBC (10ml) and mixed well. During mixing, matured chromic

chloride solution (6ml, 0.1mg/ml, pH 5.0) was added dropwise and mixing was continued for a further 10 sec. The red cell suspension was covered with PBS (1ml) and left to incubate at 4°C overnight. The supernatant was removed, the ORBC-antibody conjugate (EA) was washed twice with RPMI 1640, and then resuspended to 2.5% (v/v) in RPMI 1640. The EA conjugate was suitable for use for up to 2 weeks in this condition.

#### 7.11 Separation of rabbit T and non-T leucocytes

The isolated rabbit PBL suspension was pelleted by centrifugation at 200g for 15 min in a sterile, V-bottomed centrifuge tube. The pelleted cells were resuspended in a minimal amount of medium and mouse anti-rabbit T cell IgG in PBS was added at a ratio of 2µg of protein/10<sup>6</sup> cells. The cell suspension was incubated on ice for 30 min and then washed twice in ice-cold RPMI 1640. Equal volumes of treated rabbit PBL cell suspension (3 x 10<sup>6</sup> cells/ml) and 2.5% EA conjugate at 4°C were mixed. Aliquots (3-4mls were placed in sterile centrifuge tubes and incubated overnight at 4°C. The supernatant was removed and the cell pellet was gently resuspended, by using a sterile, glass Pasteur pipette, in fresh RPMI 1640 (2ml). The cell suspensions were combined, carefully layered (8ml) onto Ficoll-Hypaque density gradients (3ml) and centrifuged at 400g for 30 min. Non-T leucocytes were harvested from the interface with a sterile, glass Pasteur pipette, washed twice and resuspended to the

required cell concentration in RPMI 1640. The overlaying density gradient was discarded and the T lymphocytes were recovered from the red cell pellet by incubation with 0.83% (w/v)  $\text{NH}_4\text{Cl}$  - Tris buffer (10ml) (see Section 2) for 5 min at 23°C. The T cell population was washed twice and resuspended in RPMI 1640 to the required cell concentration for plating out.

Non-T leucocytes and a sample of the T lymphocytes were inactivated by irradiation (2500 rad) by using a  $^{60}\text{Co}$  gamma ray source. Culture conditions and harvesting and pulsing procedures were as described for the human AMLR cultures (Methods, p68 ).

#### 7.12 Purification of mouse-anti-(rabbit T cell) (MART-cell) immunoglobulin G from ascites fluid

The commercially obtained ascitic fluid (RIA U.K. Ltd., Tyne and Wear) was extensively dialysed against 0.04M potassium phosphate containing 0.3M NaCl, pH 8.0 (200 vol.). A DE52 column (5ml of DE52/ml of ascites) was equilibrated with the above buffer and the dialysed ascitic fluid was diluted with an equal volume of the buffer. The diluted, dialysed ascitic fluid was applied to the column and eluted with the buffer. Fractions (0.5ml) were collected and the absorbance of the solution at 280nm was measured in a UV-spectrophotometer. The fractions containing IgG

were pooled and the  $A_{280}$  of the pooled fractions was measured. The concentration of IgG present was determined by dividing the observed  $A_{280}$  of the pooled fractions by the extinction coefficient of a solution of IgG (1mg/ml).

#### 7.13 Treatment of cells with MART-cell antibody and complement

Cells were suspended in complete medium containing 10% FCS (v/v) at a concentration of  $1 \times 10^7$  cells/ml. Cell suspensions were incubated with MART cell antibody (0.5 mg/ml) at a ratio of 10 $\mu$ g antibody/ $10^6$  cells at room temperature for 20 min with occasional gentle mixing. Sterile guinea pig complement (2 vol. 1:4 dilution) was then added with shaking, and the cells were incubated at 37°C for 1h. Dead cells were removed by centrifugation (900g, 10min) of the cells through FCS (Mishell and Shiigi, 1980), and the cell pellet was treated a second time with MART-cell antibody and complement in the same way.

Control experiments using normal mouse serum (NMS) and complement were performed as for the MART-cell antibody and complement as described above.



## 8.0 RESULTS

The 'in vitro' lymphoproliferative characteristics of the rabbit immune system have not been as extensively investigated as those of mouse or man. Available data are based on the responses of the cells of the lymph nodes, spleen or thymus and other lymphoid organs (Wilson et al, 1976; Watkins et al, 1984).

The effects on the rabbit immune system resulting from the induction and maintenance of EAMG are presented.

### 8.1 The preparation of AChR from *Torpedo marmorata*

Typical results obtained during the purification of the AChR from frozen tissue at (-80°C) from the electric organs of the electric ray, *Torpedo marmorata*, are shown in table 8.1.

As shown in table 8.1, greater than 99% of the receptor present in the crude extract bound to the  $\alpha$ -toxin labelled Sepharose 4B beads. Recovery was approximately 70% in both preparations and specific activities of the purified receptor were 8.55 and 9.38 pmol/ $\mu$ g.

Table 8.1: The results of two preparations of AChR from the frozen electric organs of Torpedo Marmorata.

	<u>EXPT. 1</u>	<u>EXPT. 2</u>
FROZEN TISSUE WT. (g)	300	250
* TOXIN BINDING (CRUDE EXTRACT) (pmol/ml)	600 (200)	450 (150)
* TOXIN BINDING (NON-BOUND) (pmol/ml)	5	2
PROTEIN CONC. (DE-52 ELUATE) (ug/ml)	1500	704
+ TOXIN BINDING (FINAL ASSAY) (pmol/ml)	14071 (6)	6017 (8.1)
RECOVERY TOXIN BINDING ACT. (%)	70.4	72.2
SPECIFIC ACTIVITY (pmol/ $\mu$ g)	9.38	8.55

Numbers in brackets represent the volumes (ml) of each sample as the crude extract and DE-52 column eluate containing the major toxin binding activity.

\* Ammonium Sulphate Assay

+ DEAE Filtration Assay

## 8.2 Separation of rabbit PBL: Assay selection

The use of a 3.5% solution of Dextran in 0.9% saline cleanly removed the rabbit RBC from the whole blood sample. Following application of the leucocyte rich supernatant to a Ficoll-Hypaque gradient to remove any residual rabbit RBC, a yield of  $2.2 \pm 0.6$  (mean  $\pm$  S.D.)  $\times 10^6$  PBL per ml of whole blood was obtained, over 60 determinations.

Various methods for separating rabbit T and non-T leucocytes were investigated. Table 8.2 represents the efficacy of each of these treatments. As shown in Table 8.2 all the rosetting methods using native, enzymically or chemically-treated autologous rabbit RBC, SRBC or ORBC yielded very low numbers of rosette forming cells.

Only those ORBC which had been coupled to goat anti-mouse (GAM) antibody by the chromic chloride method of Gold and Fudenberg (1967), and then indirectly rosetted with mouse anti-(rabbit T cell) antibody (MART antibody) produced strong rosettes containing greater than 3 red cells per lymphocyte. Following separation from non-rosetted non-T cells on a Ficoll-Hypaque gradient, the rosettes yielded cell populations which responded vigorously to stimulation by Con-A. Cells at the interface (non-rosetted), on the other hand, responded very poorly to stimulation by the T cell mitogen Con-A.

The Cell Surface Markers and mitogen responses of rabbit PBL after various rosetting techniques

TABLE 8.2

METHOD	RED CELL	WHOLE PBL % MART +ve	WHOLE PBL % SmIg +ve	Con-A RESPONSE OF PELLET CELLS (cpm)	Con-A RESPONSE OF INTERFACE CELLS (cpm)
NATIVE AUTOLOGOUS	RABBIT	10	52	5025	6450
PAPAIN-TREATED AUTOLOGOUS	RABBIT	10	50	5340	6301
NEURAMINIDASE AUTOLOGOUS	RABBIT	15	51	5999	6322
2-AET AUTOLOGOUS	RABBIT	7	50	289	27499
PAPAIN-TREATED	SHEEP	2	54	202	31569
PAPAIN-TREATED	OX	0	49	0	30011
MART/GAM TREATED	OX	42	52	36256	156

KEY: MART/GAM; mouse anti-rabbit T cell/goat anti-mouse double antibody method  
 2-AET; 2-aminoethylisothiuronium bromide hydrobromide  
 PBL; Peripheral blood lymphocytes  
 SmIg + ve; Surface membrane Immunoglobulin positive cells  
 MART + ve; Mouse anti-rabbit T cell positive staining cells

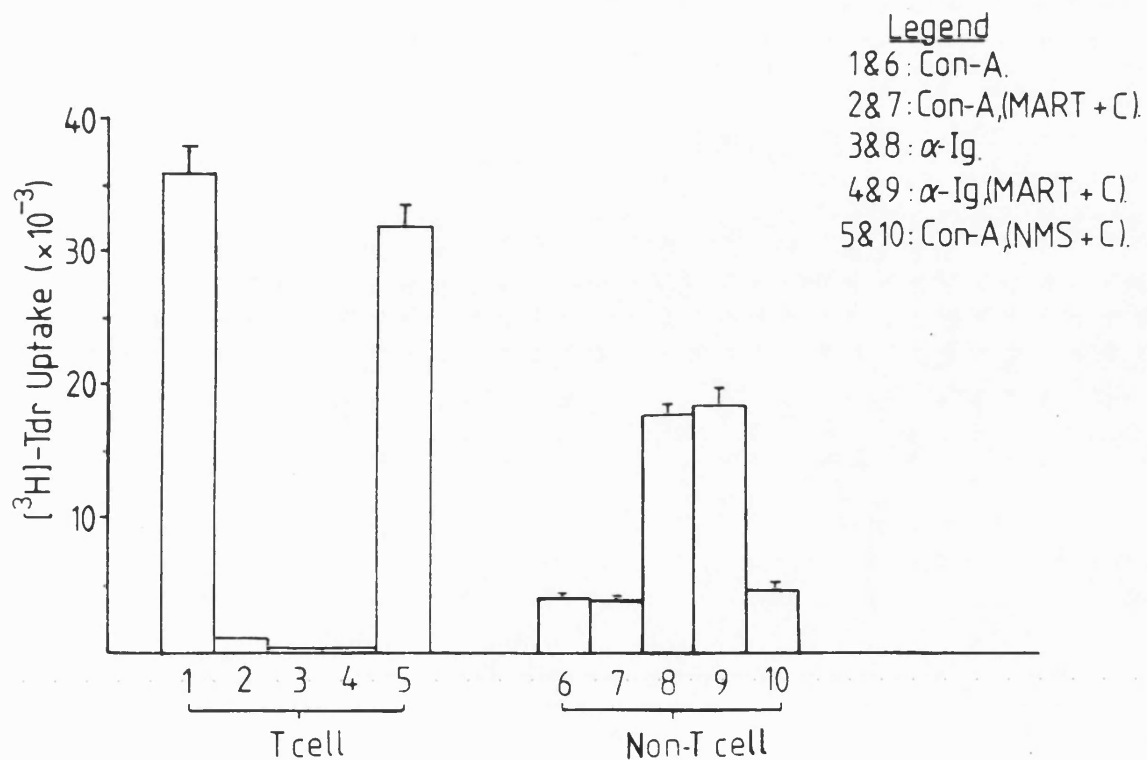
In contrast, PBL populations rosetted with either native, papain-treated or neuraminidase treated autologous RBC showed similar Con-A responses for both the interface and the pelleted cells, indicating that very little fractionation of T cells from non-T cells had occurred. Attempted cell fractionation using AET-treated autologous rabbit RBC, papain-treated SRBC, or papain-treated ORBC also showed little or no separation of T cells from non-T cells. In these cases, low Con-A inducible responses were found in the cells isolated from the cell pellet and high Con-A responses were found in the cells taken from the interface, following Ficoll-Hypaque density gradient fractionation.

In view of the above results, the indirect double antibody method was chosen for use as a means of routinely separating rabbit T and non-T cells.

### 8.3 Identification of separated cell types

In an attempt to support the results of the previous experiment, the MART/GAM-ORBC purified T cells were treated with mouse anti-rabbit T-cell antibody and complement (Methods, p<sup>196</sup>), and then tested for their ability to respond to Con-A and anti-immunoglobulin antibody. The separated non-T cells were treated similarly.

**Figure 8.1** The mitogenic proliferative responses of purified putative T cells and non-T cells to Con-A and anti-Ig ( $\alpha$ -Ig) antibody. Purified T cells ( $2 \times 10^5$  or non-T cells ( $2 \times 10^5$ ) were incubated for 4 days in complete medium containing 10% FCS (v/v) and sub-optimal concentrations of Con-A ( $4\mu\text{g/ml}$ ) or anti-Ig ( $50\mu\text{g/ml}$ ). Each bar represents the mean response of three individual normal rabbit T-cell and non-T cell populations  $\pm$  s.e.m. Pre-treatment with MART cell antibody plus complement or normal mouse serum (NMS) plus complement was performed as described (Methods, p196 ).



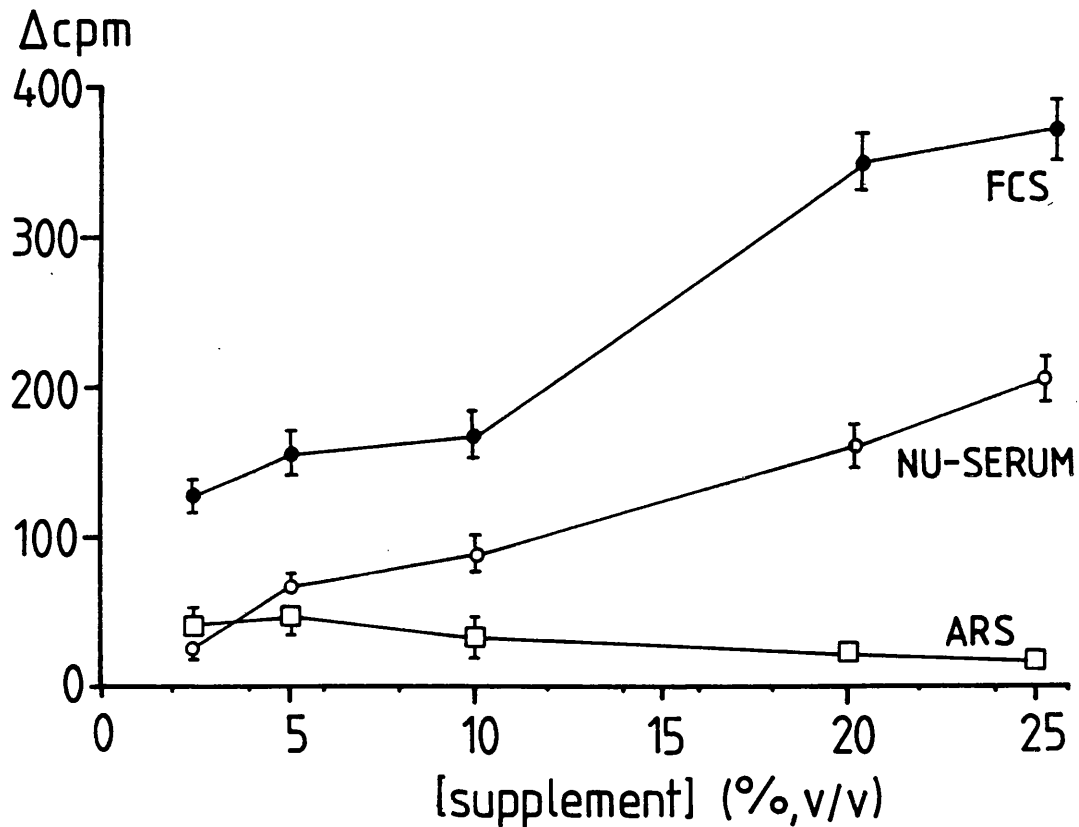
As shown in figure 8.1, treatment of the purified T cells with MART antibody plus complement resulted in complete abrogation of the Con-A induced T cell response. This, together with the completely absent response to anti-Ig antibody treatment indicates that the MART/GAM-ORBC rosetting population is indeed the T cell population. Treatment of the T cells with normal mouse serum plus complement did not affect the mitogenic responses of the T cells, and no changes in the non-T cell mitogenic responses were observed as a result of the anti-T cell antibody plus complement treatment.

#### 8.4 The development of culture conditions for the rabbit AMLR

Following the establishment of a satisfactory method for the separation of T and non-T cells, the conditions required for optimal cell proliferation in the normal rabbit AMLR were determined.

Preliminary studies showed only a low proliferative response in the rabbit AMLR and enhancement of the AMLR by optimisation of the culture conditions was considered essential. The effects of FCS, NU-SERUM, and autologous rabbit serum as culture medium supplements were accordingly investigated.

Figure 8.2 shows the effect of 2.5% - 25% serum supplementation on the AMLR responses.



**Figure 8.2** The effect of FCS, NU-SERUM and ARS on the AMLR proliferative response. Rabbit T cells ( $1 \times 10^5$ ) and irradiated (2500 rad) autologous rabbit non-T cells ( $1 \times 10^5$ ) were incubated at  $37^\circ\text{C}$  for 9 days in complete medium ( $200\mu\text{l}$ ) containing FCS, NU-SERUM, or ARS at concentrations of 2.5%, 5%, 10%, 20% and 25% (v/v). Each point represents the mean of three individual rabbit PBL responses  $\pm$  s.e.m.



The proliferative responses observed in the presence of a range of concentrations of autologous rabbit serum (ARS) were substantially lower than those in the presence of NU-SERUM or FCS. Of the latter two supplements, FCS gave the higher responses and so was chosen for subsequent experiments.

#### 8.5 The effect of cell concentration and ratio of T- to non-T cells

T cells at concentrations of  $0.5 \times 10^6$  cells/ml,  $1.0 \times 10^6$  cells/ml, and  $2.0 \times 10^6$  cells/ml were incubated with non-T cells at concentrations between  $0.5 \times 10^6$  cells/ml and  $2.5 \times 10^6$  cells/ml, as shown in figure 8.3. The combinations of the T cells and non-T cells at these concentrations yielded T cell to non-T cell ratios of between 1:5 and 4:1.

Optimal proliferative responses were yielded by T cells and non-T cells at equal concentrations, both at  $1 \times 10^6$  cells/ml and  $2 \times 10^6$  cells/ml. The cultures containing  $0.5 \times 10^6$  cells/ml of T cells generally yielded lower AMLR responses.

Thus, for routine rabbit AMLR cultures, T cells and non-T cells both at cell concentrations of  $1 \times 10^6$  cells/ml, in complete medium containing 20% FCS, were employed.

Figure 8.3 The effect of different cell concentrations and ratios of T cells to autologous, irradiated (2500 rad) non-T cells on the rabbit AMLR. T cells (100 $\mu$ l) at concentrations of  $0.5 \times 10^6$  cells/ml,  $1.0 \times 10^6$  cells/ml, and  $2.0 \times 10^6$  cells/ml (arrows) and irradiated (2500 rad), autologous non-T cells (100 $\mu$ l), at concentrations between  $0.5 \times 10^6$  cells/ml and  $2.5 \times 10^6$  cells/ml, were incubated in complete medium containing 20% FCS (v/v) at 37°C for 9 days.

Each point represents the mean, peak proliferative AMLR response ( $\pm$  s.e.m.) of cells from three normal rabbits. Each individual rabbit AMLR response was represented by the mean of quadruplicate AMLR cultures minus the sum of the AMLR of T cells cultured with irradiated (2500 rad), autologous T cells and that of irradiated (2500 rad), autologous non-T cells alone.

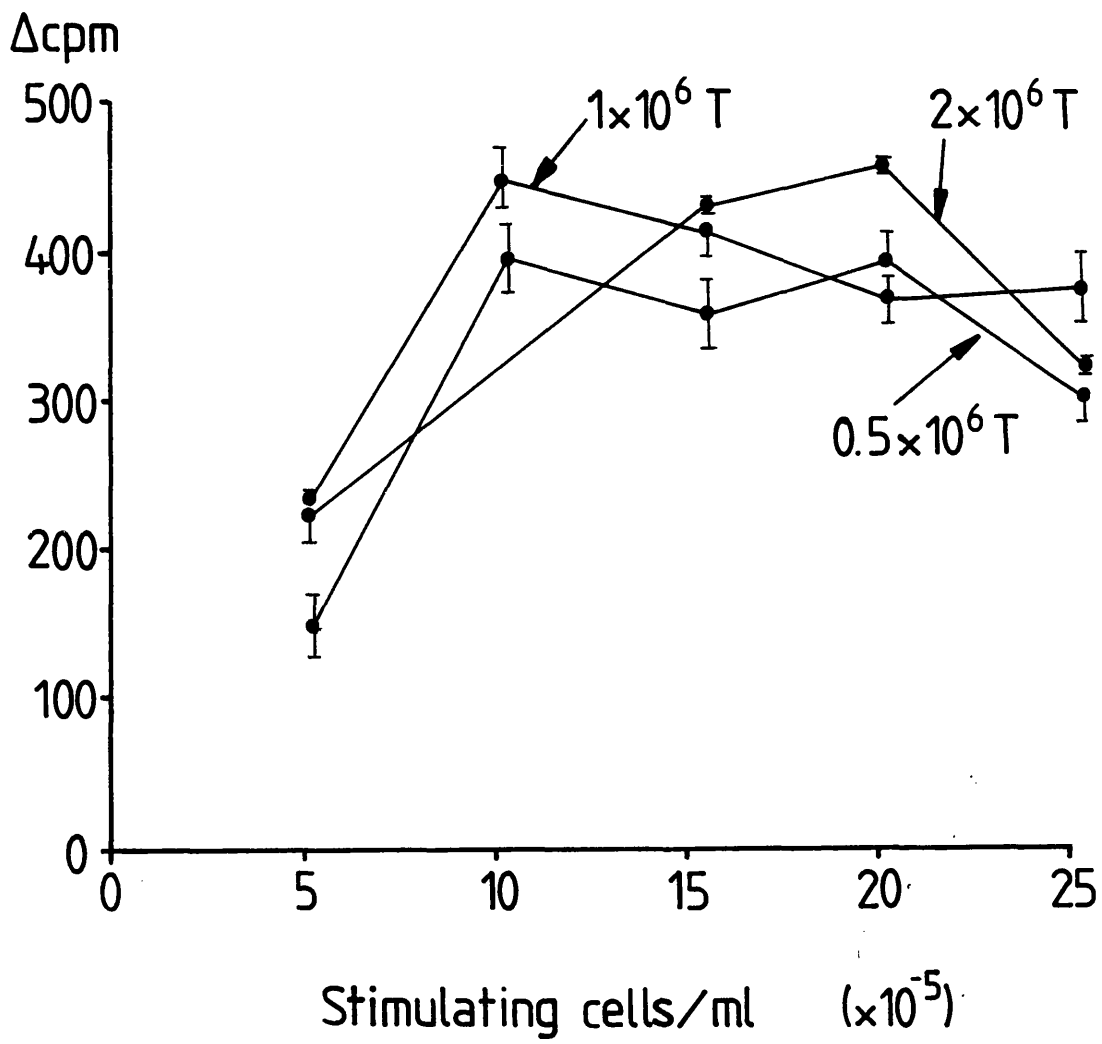


Figure 8.3

## 8.6 AMLR in normal and EAMG rabbits

The rabbit AMLR presents a much lower proliferative response than that observed in the human equivalent, ranging between 252  $\Delta$ cpm and 452  $\Delta$ cpm ( $359 \pm 10.6$ , 39; mean  $\pm$  s.e.m., n) for the normal rabbit. Figure 8.4 shows the scatter of normal, pre-immunisation responses and post-immunisation responses.

The kinetic AMLR response for normal rabbits shown in figure 8.5 (a) resembles the AMLR proliferative response observed in the normal human PBL population. However, as already noted the magnitude of the rabbit AMLR response is dramatically reduced in comparison to that observed in the human. Also, the peak AMLR response appears to occur slightly earlier than the human peak AMLR response, appearing rapidly between days 5 and 6 rather than gradually between days 4 and 7.

Figure 8.6 shows the kinetic AMLR response of a rabbit with EAMG. This response was considered to be representative of the kinetic AMLR responses shown by myasthenic rabbits and has a profile which is different in many ways to that seen in normal rabbits. Very little proliferation occurs before day 5 of the culture period and no distinct, rapid increase is observed after this time point. A small, gradual increase in the proliferative response occurs, with a plateau

Figure 8.4 A scatterplot of the peak AMLR proliferative responses observed between T cells ( $1 \times 10^5$ ) and irradiated (2500 rad), autologous non-T cells ( $1 \times 10^5$ ), for six control rabbits and for rabbit numbers 57-62 at the various stages of the immunisation procedures. Each point represents the peak AMLR proliferative response during the 9 day culture period, irrespective of the day on which it occurred. Each point represents the mean of quadruplicate AMLR cultures. The horizontal bars represent the means of each data group and the symbols (  $\square$  /  $\sqcap$  ) represent the s.e.m.

Fig.8.4

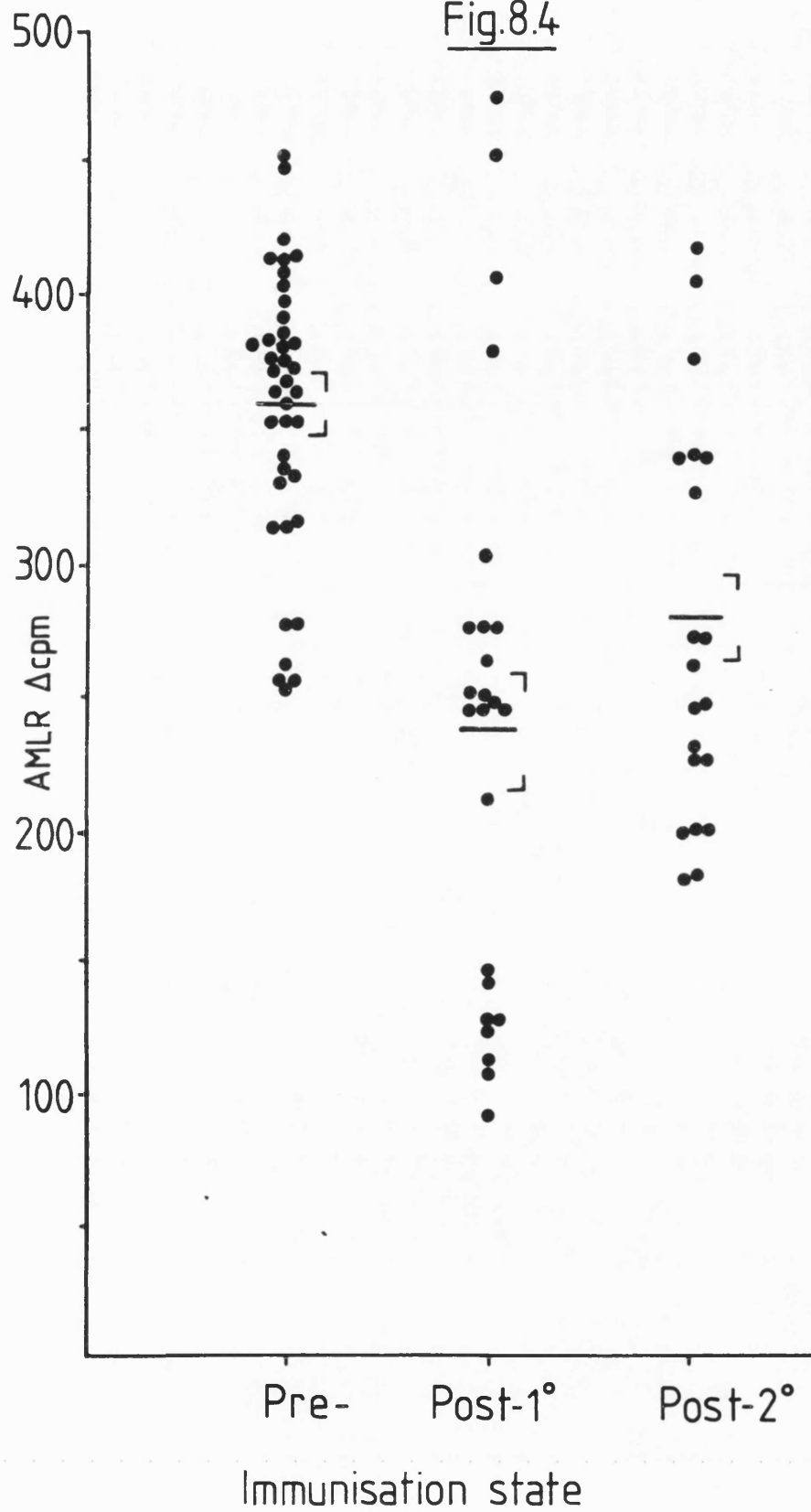


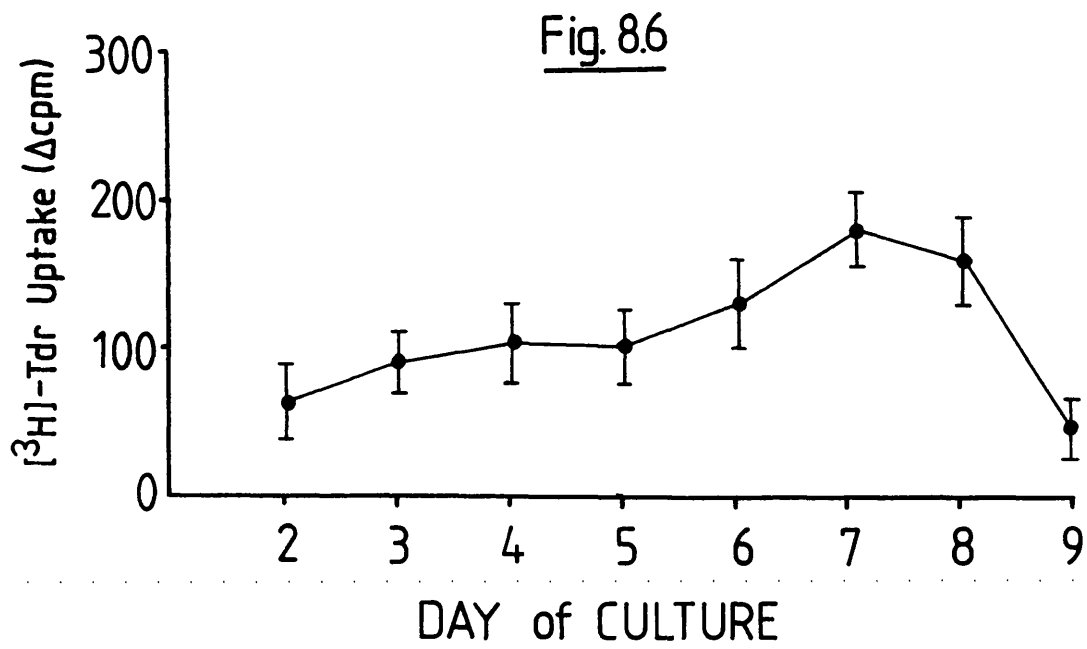
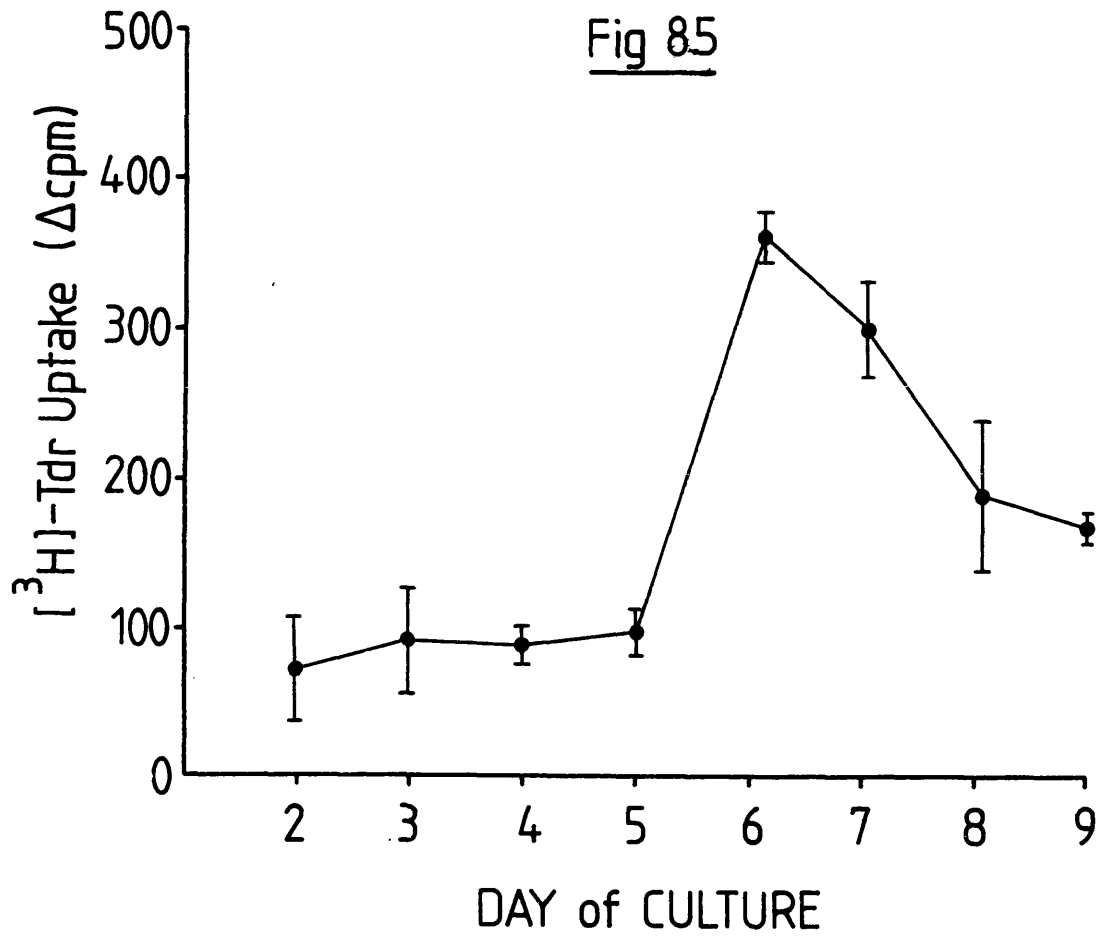
Figure 8.5    The kinetic AMLR response of

- a)    Normal    rabbit    T    lymphocytes    ( $1 \times 10^5$ )    and  
autologous, irradiated (2500 rad) non-T leucocytes  
( $1 \times 10^5$ ) in complete culture medium (200 $\mu$ l).

Figure 8.6

Myasthenic rabbit T lymphocytes ( $1 \times 10^5$ ) and  
autologous, irradiated (2500 rad) non-T leucocytes  
( $1 \times 10^5$ ) in complete culture medium (200 $\mu$ l).

Each point represents the mean of quadruplicate  
AMLR cultures  $\pm$  s.e.m.





rather than a peak at the time of the maximal proliferation. This plateau occurs later than the peak observed in normal rabbits and decreases to a lower value on day 9 than its normal counterpart. Overall, the profile is flat and the peak response is of a low magnitude for the AMLR in rabbits with EAMG.

## 8.7 Longitudinal studies of immunised rabbits

The effects of the induction and maintenance of EAMG on the humoral and cellular immune responses of rabbits 57-62 are shown in figures 8.7 - 8.12. The upper diagram (a) of each figure represents the anti-AChR antibody titre development and the longitudinal monitoring of the AMLR. The lower diagram (b) of each figure represents the mitogen-induced cellular proliferative responses for Con-A and PHA-P at sub-optimal concentrations.

### 8.7.1 Anti-AChR antibody titres and physical state

The humoral responses to the immunisation with solubilised AChR from Torpedo marmorata appear to fall into two groups.

- i) those rabbits which develop substantial titres following the initial antigenic challenge and,

- ii) those rabbits which develop substantial titres only after the second, 'booster' immunisation.

The former group consists of rabbits 58, 59 and 60, all of which had post-primary immunisation serum titres of at least 30nM anti-AChR antibody. Detectable anti-AChR antibody levels were present 3 weeks after the initial immunisation in all three of these rabbits.

A peak anti-AChR antibody level occurred 6 weeks after the initial immunisation in all of the rabbits except rabbit 62, which showed a peak at week 5. Following the booster immunisation in week 7, substantial responses were detectable in all of the rabbits. However, rabbits 58 and 59 which had previously exhibited substantial humoral responses following the initial immunisation, rapidly developed acute EAMG and had to be destroyed. Rabbits 57, 60, 61 and 62 gradually developed substantial humoral responses of the order of 30-60nM serum anti-AChR antibody. Clinically, these four animals exhibited the classical characteristics of EAMG in rabbits, drooping neck and ears and slight paralysis of the fore and hind limbs. Loud respiratory noises may have been due to laryngeal paralysis and these often disappeared when muscular strength was regained. Clinical signs were not apparent until after the second immunisation in any of the rabbits.

The continually increasing humoral response following the secondary immunisation in rabbits 57, 60 and 61 did not result in such a marked decline in these animals' well-being, symptoms being restricted to drooping neck and ears.

#### 8.7.2 The AMLR

AMLR responses for those rabbits in which EAMG was induced were studied over a 16 week period (Figs. 8.7 - 8.12). Each point represents the peak proliferative response during a nine day AMLR culture. Cellular responses were studied for each animal at two-weekly intervals to allow the animal to recover from the cardiac puncture procedure.

No significant differences could be seen in the AMLR responses from week to week for any individual rabbit, except for rabbit 62. Following a slight increase in the somewhat lower than normal AMLR response at week 4, a substantial decrease in the AMLR occurred between week 10 and week 12. This corresponded with a marked decline in the physical well-being of rabbit 62, paralysis of the hind limbs and an apparent loss of appetite being notable at this time.

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All of the rabbits exhibited AMLR over the longitudinal study which were significantly different from those observed in the normal group. Figure 8.4 shows a scatterplot of all the pre-immunisation (including control rabbits) AMLR responses and those AMLR responses observed following the primary and secondary immunisations.

The mean peak AMLR response following the initial immunisation are statistically significantly decreased ( $P < 0.04$ ) whereas the mean peak AMLR response following the second immunisation procedure was not significantly decreased. Rabbits 57 (Figure 8.7 (a)), 59 (Figure 8.9 (a)), and 61 (Figure 8.11 (a)) all exhibited AMLR responses which gradually increased towards normal, pre-immunisation levels following the significant initial decrease. Rabbits 58 (Figure 8.8 (a)) and 60 (Figure 8.10 (a)) both exhibited significantly reduced AMLR which remained low throughout the study period.

### 8.7.3 Proliferative responses of rabbit T cells

Concurrent with the monitoring of the humoral and AMLR responses, the responses of rabbit T cells to the mitogens Con-A and PHA-P were studied. These responses are shown in Figs. 8.7-8.12.

As with the AMLR, the proliferative responses of rabbit T cells to the mitogens Con-A and PHA-P were substantially lower than those observed for human T cells. In contrast to the responses observed with human T lymphocytes, the responses induced by Con-A were greater than those induced by PHA-P. The PHA-P responses were generally unaffected by the induction and maintenance of EAMG. Con-A-induced responses showed greater variation during the period of study, the most dramatic changes being observed for rabbits 58 and 62. For rabbit 58, a rapid decline in the response following the initial immunisation did not correlate with changes in any of the other cellular responses, although it did accompany an increase in anti-AChR antibody levels.

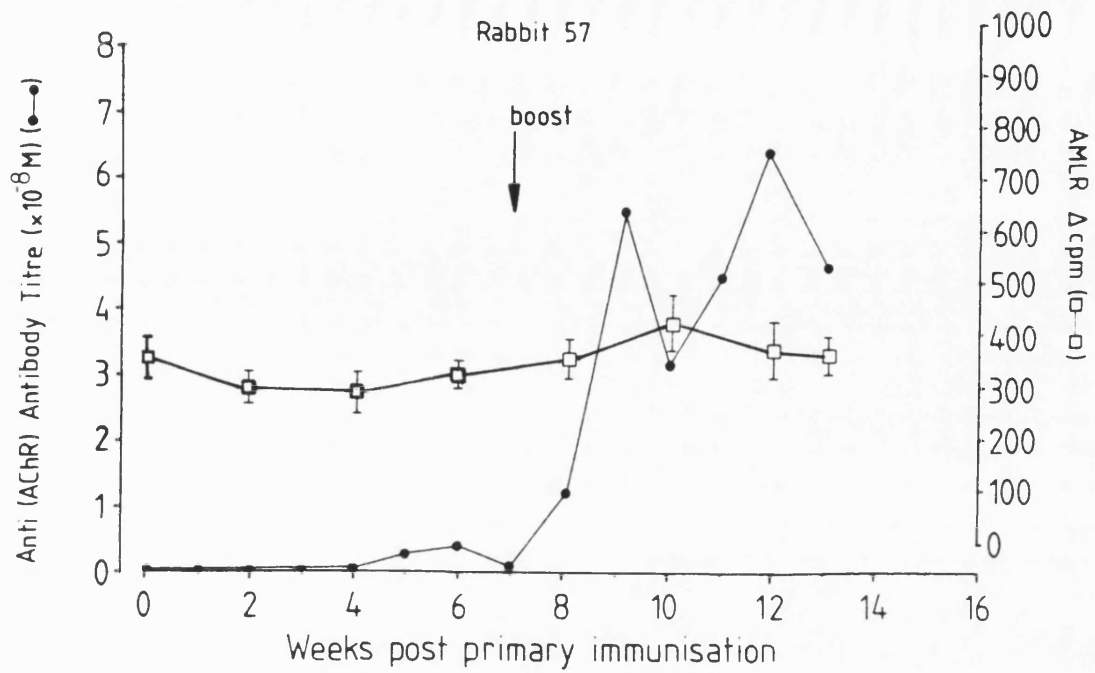
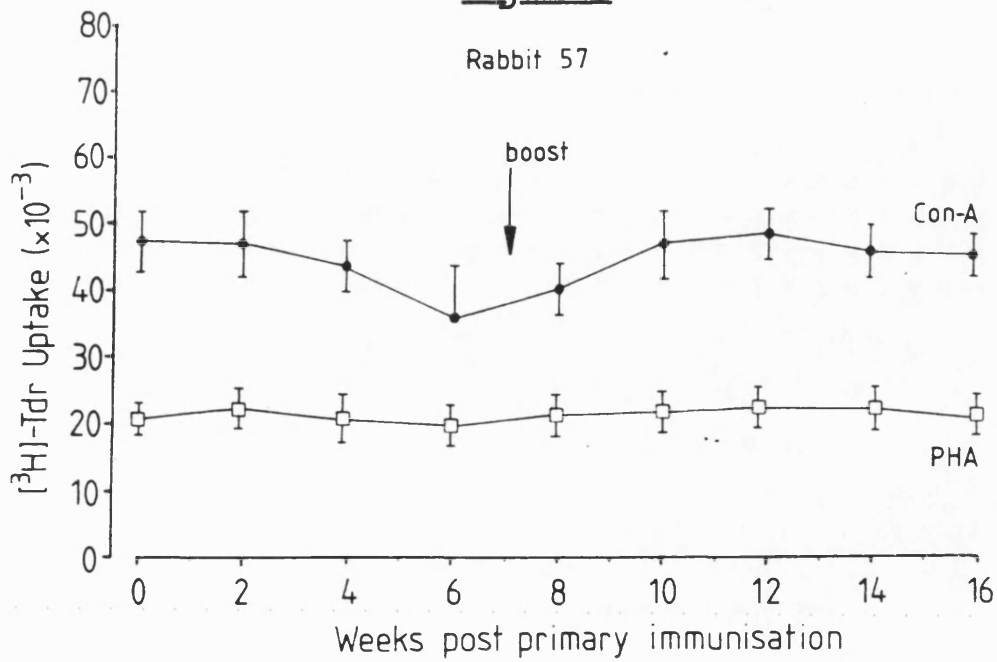
For rabbit 62, a rapid decline in Con-A induced responsiveness occurred following the second AChR immunisation, again concomitant with an increase in anti-AChR antibody levels. At a later stage in the study period, the AMLR responses of rabbit 62 also decreased.

Despite occasional fluctuations, the T cells isolated from the PBL of rabbits 57, 59 and 61 did not show any dramatic changes in mitogen induced responsiveness during the study period. This was reflected in the stability of the AMLR response throughout this period despite variations in levels of anti-AChR antibodies.

Figures 8.7 - 8.12

- a) Longitudinal studies of the anti-AChR antibody titre ( ●—● ) and the AMLR response ( □—□ ) and,
- b) the longitudinal studies of the Con-A response ( ●—● ) and the PHA-P response ( □—□ ) in normal rabbits following immunisation with Torpedo AChR in CFA and a booster immunisation of Torpedo AChR in IFA.

The AMLR, Con-A and PHA-P responses represent the means of quadruplicate cultures  $\pm$  s.e.m. The anti-AChR antibody titre represents the means of duplicate assays.

Fig.8.7 aFig.8.7 b

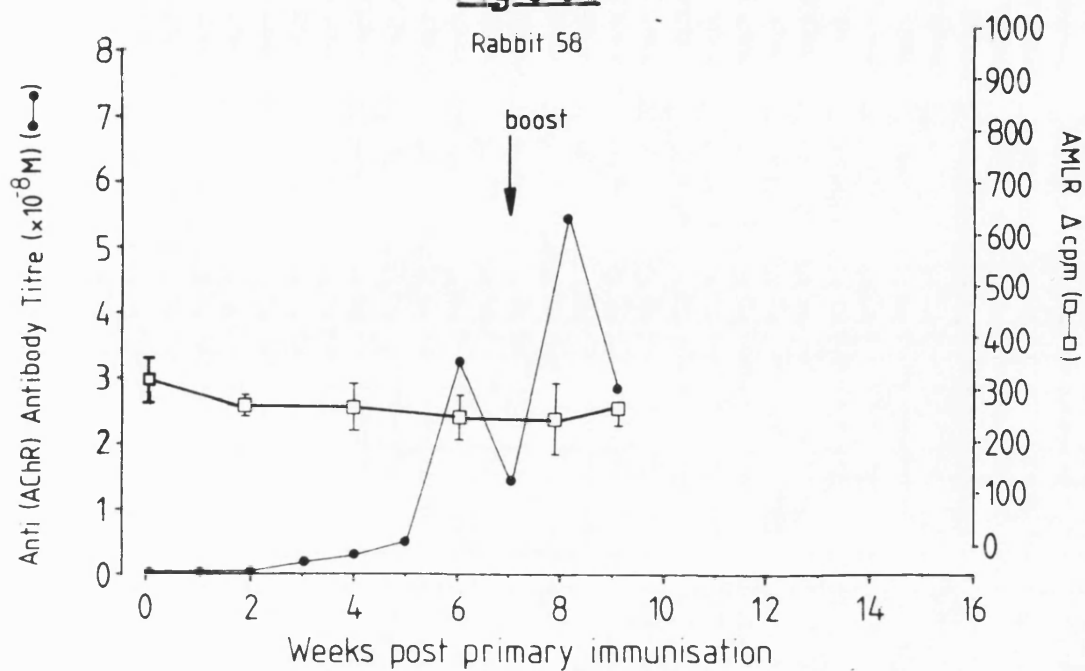
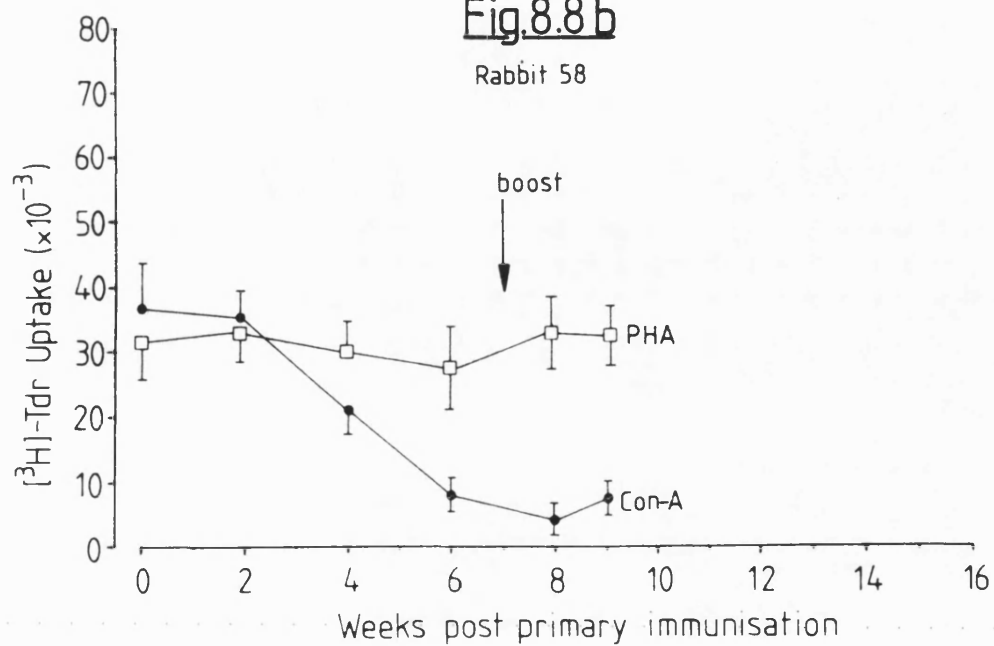
**Fig.8.8a****Fig.8.8b**



Fig.8.9 a

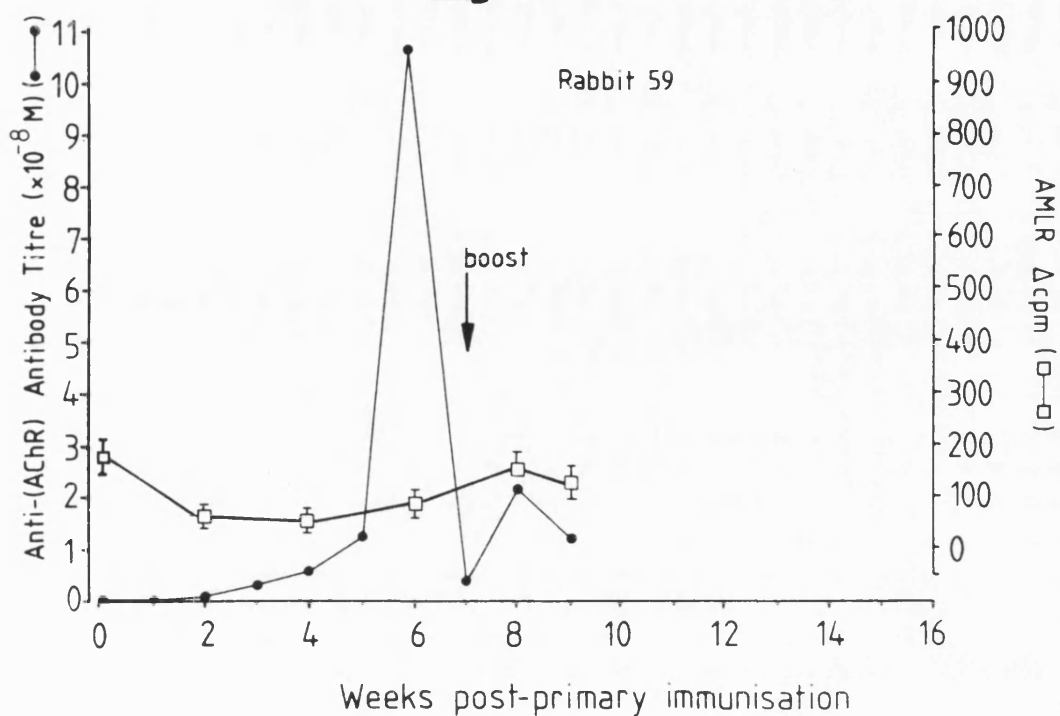


Fig.8.9 b

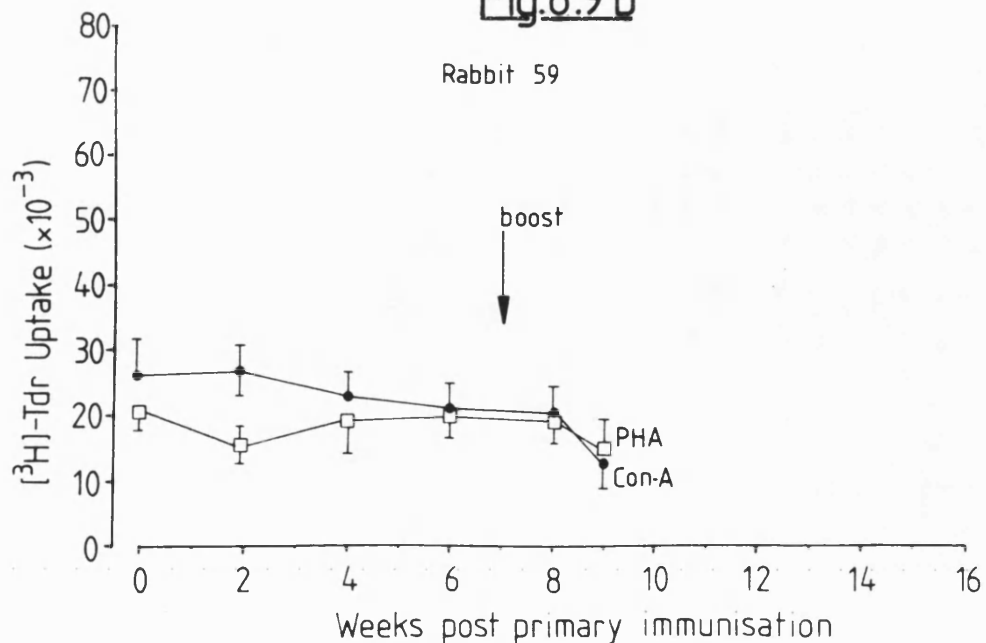


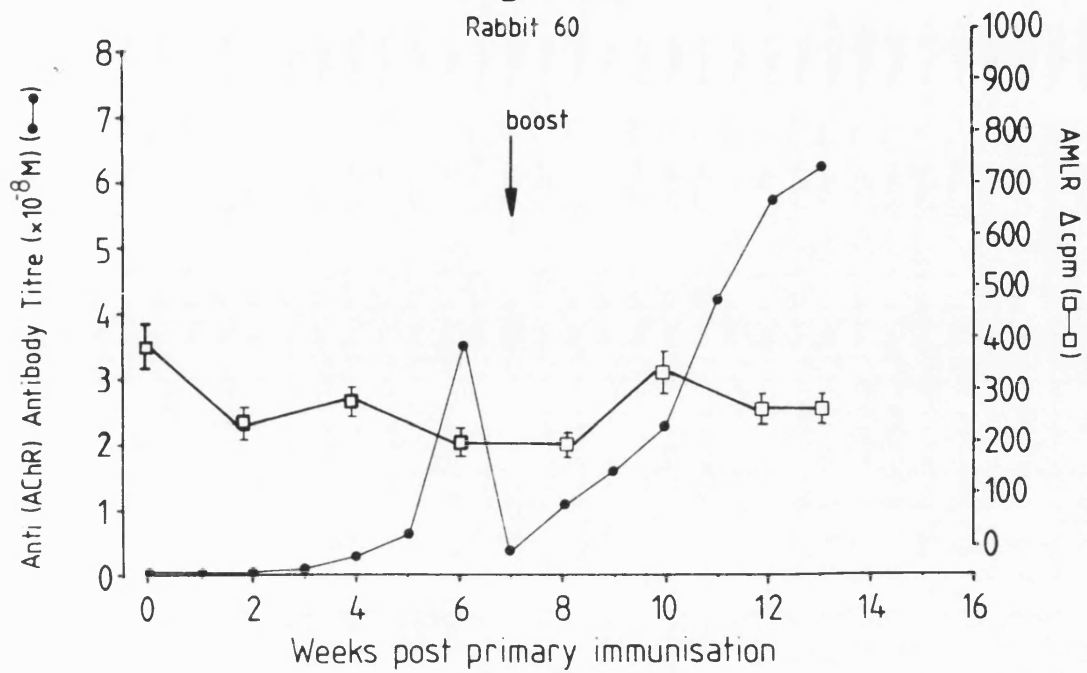
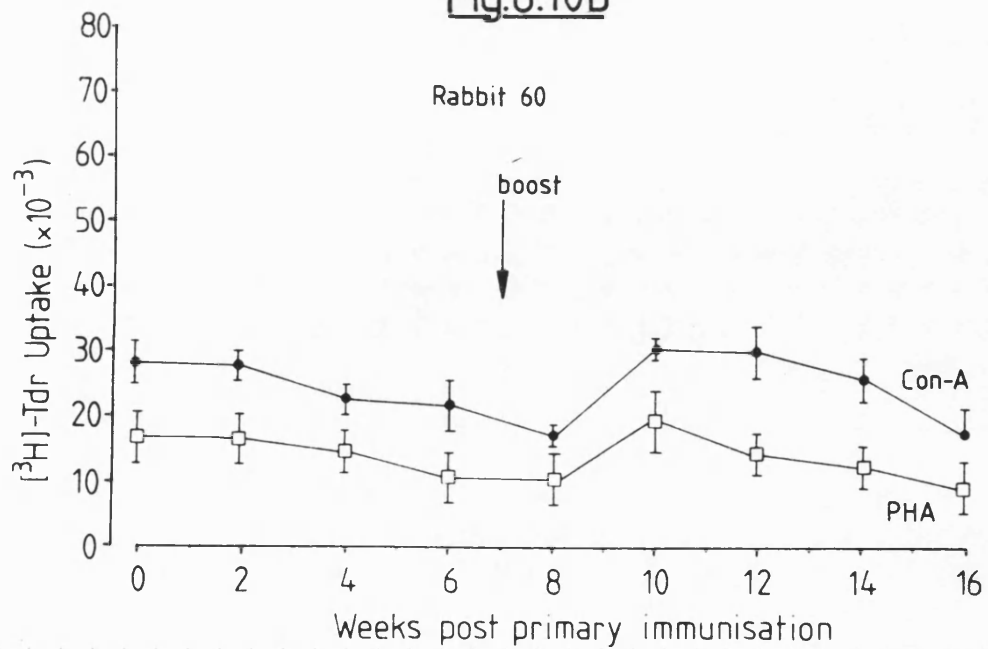
Fig.8.10aFig.8.10b

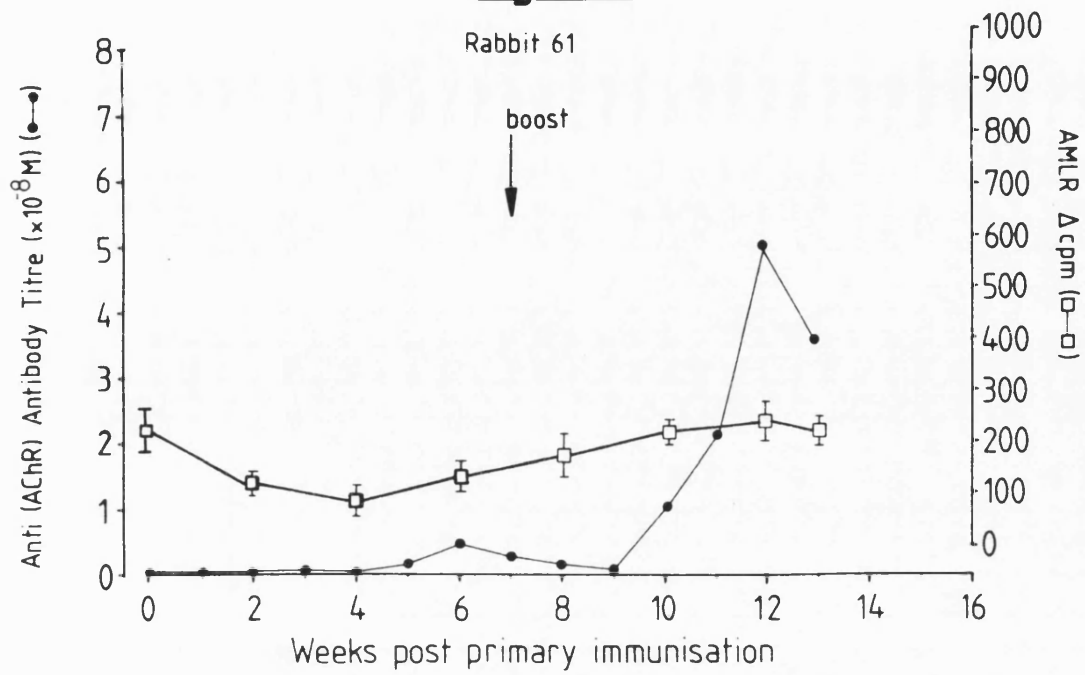
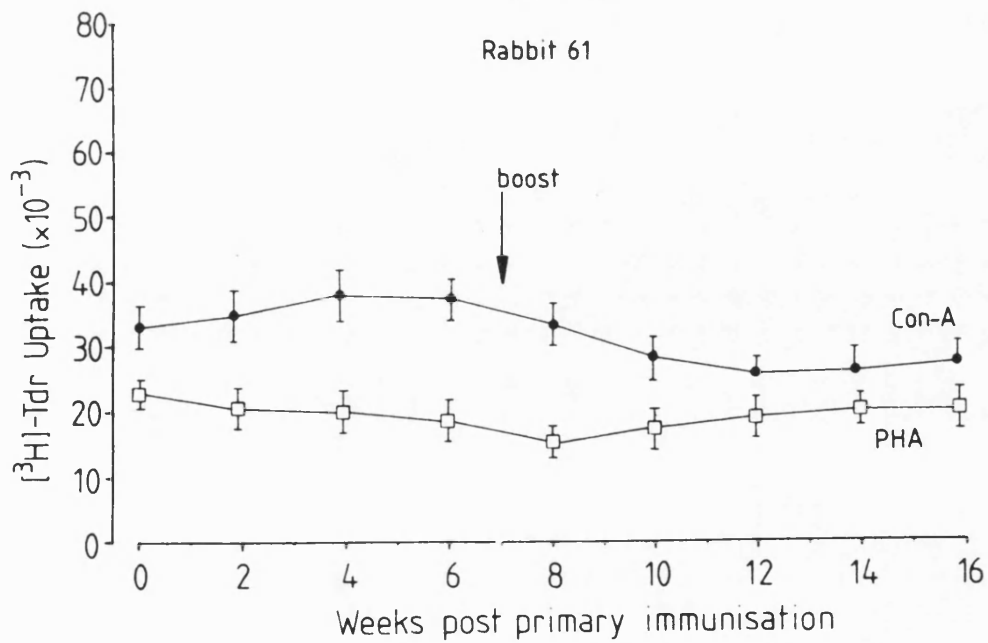
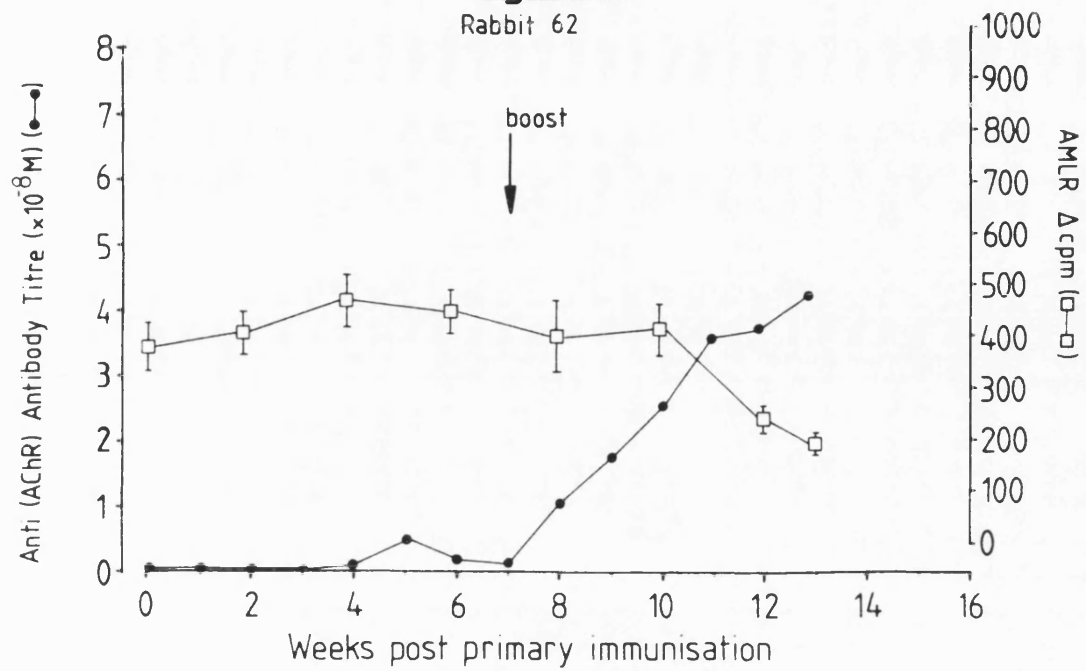
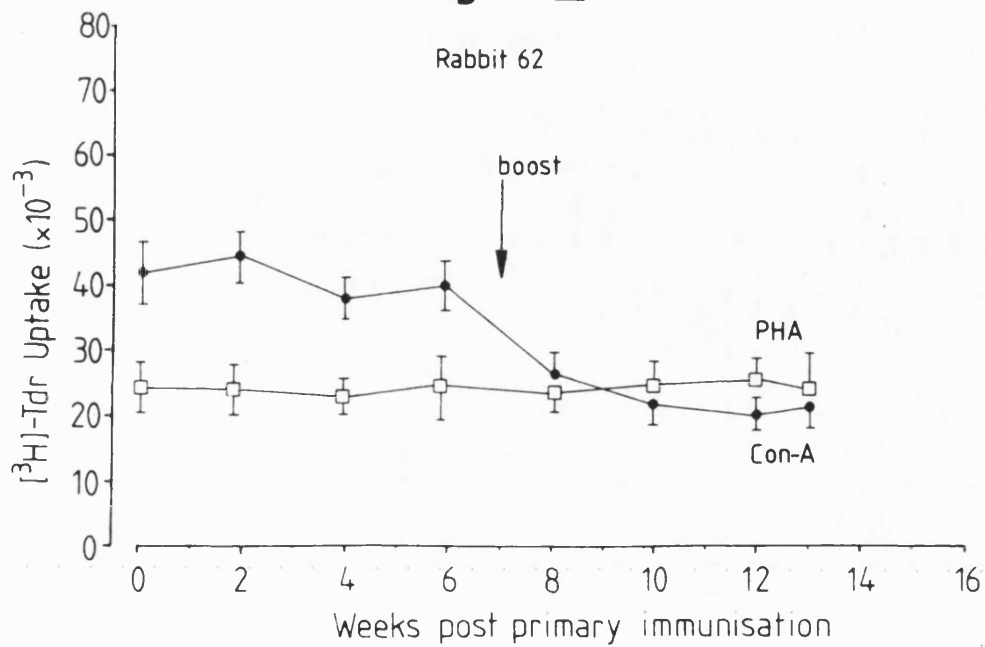
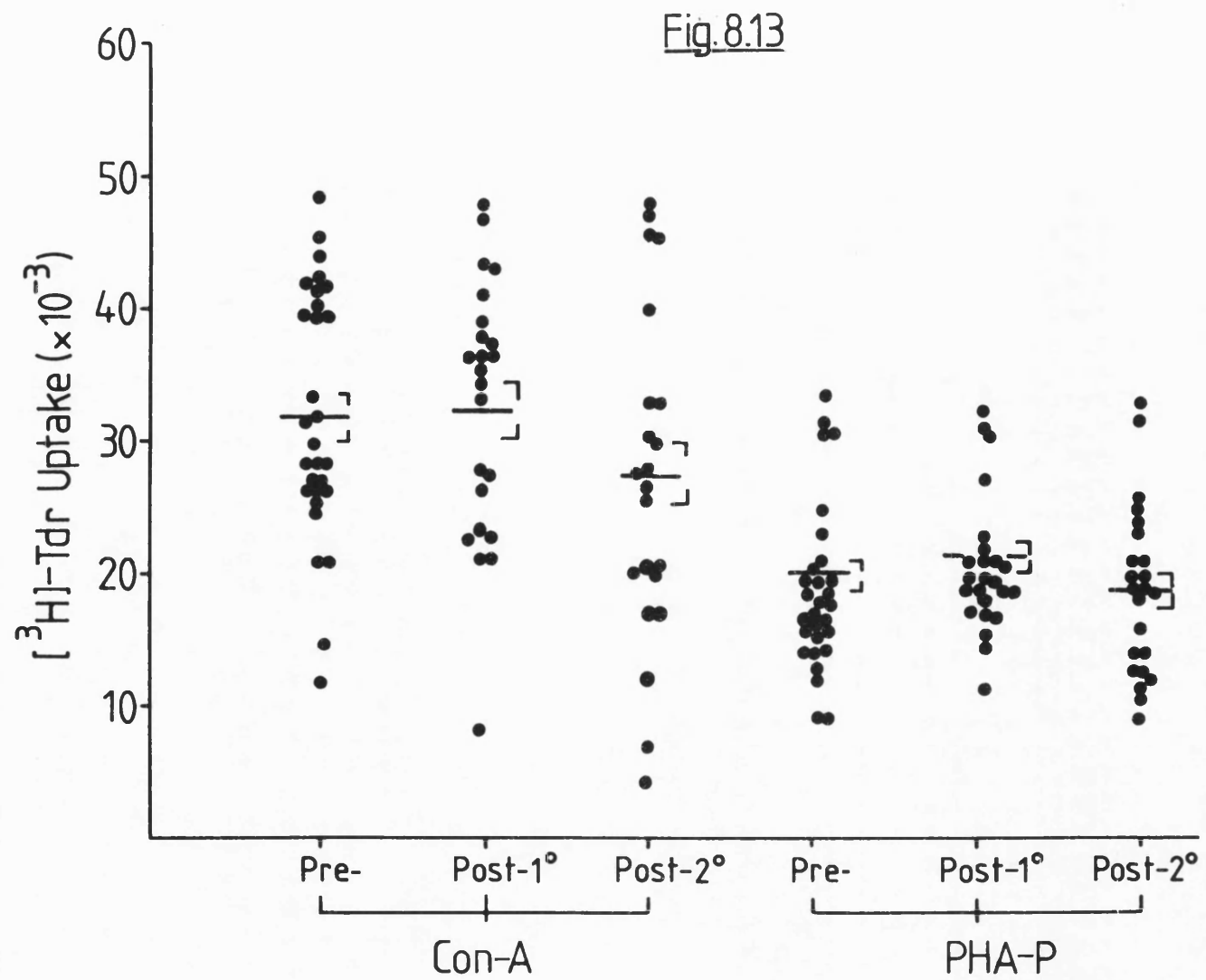
Fig.8.11aFig.8.11b

Fig.8.12aFig.8.12b

As shown in figure 8.13, no significant differences were observed between the pre-immunisation and post-primary or post-secondary immunisation Con-A induced responses of rabbit T cells. The pre-immunisation Con-A induced responses ranged from 13,500  $\Delta$ cpm to 48,500  $\Delta$ cpm (31,900  $\pm$  1,720, 29; mean  $\pm$  s.e.m., n) whilst the post-primary immunisation responses ranged from 8,000  $\Delta$ cpm to 47,500  $\Delta$ cpm (32,300  $\pm$  2,040, 23; mean  $\pm$  s.e.m., n) and the post-secondary immunisation responses ranged from 4,011  $\Delta$ cpm to 48,051  $\Delta$ cpm (27,041  $\pm$  2,444, 22; mean  $\pm$  s.e.m., n).

Similarly, no significant differences were observed between the pre-immunisation and post-primary or post-secondary immunisation PHA-P induced responses of rabbit T cells. The pre-immunisation PHA-P induced responses ranged from 9,000  $\Delta$ cpm to 33,989  $\Delta$ cpm (19,005  $\pm$  1,221, 29; mean  $\pm$  s.e.m., n) whilst the post-primary immunisation responses ranged from 11,250  $\Delta$ cpm to 32,500  $\Delta$ cpm (21,400  $\pm$  1,506, 24; mean  $\pm$  s.e.m., n) and the post-secondary immunisation responses ranged from 9,240  $\Delta$ cpm to 33,003  $\Delta$ cpm (18,751  $\pm$  1,340, 23; mean  $\pm$  s.e.m., n).

Figure 8.13 A scatterplot of the mitogen-induced responses observed during the pre-, post-primary (post-1°), and post secondary (post-2°) immunisation periods. Sub-optimal concentrations of concanavalin-A (Con-A) (4µg/ml) and phytohaemagglutinin-P (PHA-P) (0.1%) were incubated with rabbit T cells ( $2 \times 10^5$ ) in complete medium (200µl) containing FCS (10% v/v). Each point represents the peak mitogen-induced response observed during a 5 day culture period, irrespective of the day on which it occurred. The horizontal bars represent the means of each group and the symbols ( ▮ / ▮ ) represent the s.e.m.



## 9.0 DISCUSSION

The ability to induce and maintain an animal model of MG does not guarantee the elucidation of the immunoregulatory changes that may occur during the development of this autoimmune state. The comparisons with the autoimmune state in man may also be limited. However, EAMG has provided a useful model for advances in therapeutic drug development and has enabled the confirmation of the hypothesis that MG is an autoimmune or immunopathologically mediated disease (Lisak and Zweiman, 1975). EAMG has proved to be an excellent model of the effects of the autoimmune response to AChR on neuromuscular transmission in MG, but investigation of the events that induce this response has been less successful.

Some of the immunoregulatory events in EAMG in vitro have been defined. These include antigen presentation to T helper cells by macrophages, T helper stimulation of primed B lymphocytes to antibody production and the observation that in vitro, the AChR-responsive T cell is both phenotypically and functionally a T helper cell (De Baets et al., 1982). The immune response to AChR in EAMG has been shown to be thymus dependent (Lennon et al., 1976), neither a full anti-AChR antibody response nor the development of weakness will occur in the absence of a thymus gland or T cells. One of the most critical questions that remains unanswered is what provides the primary and



sustaining stimuli in human MG and how may endogenous control mechanisms be manipulated to suppress this anti-self response. Studies on the mechanisms regulating the immune responses to AChR in EAMG may provide approaches to establishing the cause and possible cure of MG.

Despite the wealth of immunological data that exists for the murine model of EAMG, and the established disease induction and maintenance protocol that exists for the rat model of EAMG, the rabbit model of EAMG was chosen for study. The rabbit model was chosen primarily because sufficient PBL could be obtained from each animal for a repetitive assay of the cellular immune responses. This was considered to be essential if comparisons with the human condition in MG were to be drawn.

#### 9.1 Induction and maintenance of EAMG

Rabbits are one of the best species of animals available for the production of precipitating antibodies and are known to develop often fatal forms of EAMG. An intramuscular route for the immunisation of the emulsified AChR antigen was used, this being the route of choice for CFA. Previously published protocols involved subcutaneous or intradermal immunisation routes for rats and guinea pigs (Lennon et al., 1975), but CFA injected via those routes will cause ulceration of the site within 2-3 days and the loss of the antigen depot. The induction of EAMG was

monitored by using the serum anti-AChR antibody titre. No clinical signs developed in any of the AChR-immunised rabbits until after the 'booster' immunisation.

Of the six rabbits in which EAMG was induced, two animals (numbers 58 and 59) had to be destroyed following the sudden development of muscular paralysis and breathing difficulties. Both of these rabbits exhibited high anti-AChR antibody titres shortly before the onset of the clinical signs. This observation is similar to that of Elfman (1984), who reported precipitous death in rabbits with EAMG, as a result of weakness of the lung muscles which led to pneumonia. However, rabbits 57, 60, 61 and 62 also developed substantial anti-AChR antibody titres following the booster immunisation and survived, despite showing the classical signs of EAMG, such as paralysis of the hind limb muscles, drooping of the neck and ears, and loud respiratory noises. The rapidly developing humoral responses following the booster immunisation appeared to plateau during the latter stages of the study.

Thus, EAMG (as indicated by anti-AChR antibody titres and clinical signs), was induced in rabbits and maintained for six weeks. This was achieved by using AChR in adjuvant in dosages of 80 $\mu$ g (400pmol toxin binding activity) for initial immunisations and 50 $\mu$ g (250pmol) for booster immunisations. These levels of

AChR protein are relatively low in comparison to those commonly used in rats and mice (200 $\mu$ g) (De Baets et al., 1982; Christadoss et al., 1983a), which may account for the disease maintenance in an animal model which is noted for its difficulty in this respect (Elfman, 1984).

## 9.2 Cell separation

The investigation of cellular immune responses in any animal model may require the fractionation of PBL subsets. Indeed, the study of the AMLR necessitates the separation of non-T and T lymphocytes. Thus, in order to compare the AMLR responses of normal rabbits and myasthenic rabbits with those of normal and myasthenic humans, the separation of rabbit T and non-T leucocytes must be attained.

The separation of rabbit T cells from non-T leucocytes has been effected by using a rosette forming reaction between rabbit T cells and papain-treated autologous erythrocytes (Wilson et al., 1975a), similar to that observed between human T cells and sheep erythrocytes (Jondal, 1972). The report of Wilson et al., (1975b) claimed increased strengths of rosetting and increased numbers of erythrocytes bound per lymphocyte when the erythrocytes were pre-treated

with papain. However, in the present study, use of identical conditions for the treatment of autologous erythrocytes and rabbit PBL gave no results comparable to those published by Wilson et al., (1975b). Table 8 shows the various treatments of both autologous and heterologous erythrocytes and their subsequent rosetting characteristics. As shown in this table only 10% of the whole leucocyte population rosette with papain-treated autologous RBC, the same percentage that rosette with native RRBC. Indeed, neuraminidase treated RRBC bind in greater numbers to rabbit T cells than do papain treated RRBC.

The most effective method of separation of rabbit T cells from rabbit non-T cells utilised an indirect rosette technique. Mouse anti-rabbit T (MART) cell antibody was used to identify rabbit T cells, and goat anti-mouse IgG coupled ORBC were used to rosette and select out the T cell population. Using this protocol, a Con-A responsive, MART cell positive staining population was fractionated from the PBL population. Abrogation of the Con-A responsiveness of this T cell population was accomplished by treating the selected cells with MART cell antibody plus complement.

Despite the use of the same antibody for identification and separation purposes, the mitogen responses (and their abrogation as described above) and the selection of a cell population whose proportion

corresponds to that of the T cell population (see page 200 ), would appear to support the view that this separated population was the T cell population.

### 9.3 Culture conditions

In general, it has been found that rabbit leucocytes respond less vigorously or not at all in the MLR when compared to the responses of the cells of other organs, notably the spleen (Knight et al., 1971; Ozer and Waksman, 1974; Ling and Kay, 1975). However, Milthorp and Richter (1979) presented evidence that rabbit leucocytes responded well in the MLR and that an autologous MLR could be induced with modifications to the culture conditions.

Preliminary studies indicated that problems were associated with measuring the AMLR proliferative responses in rabbits. In an attempt to overcome these problems, the culture conditions were modified in terms of serum supplementation. The culture medium recommended in the report of Milthorp and Richter (1979) (RPMI 1640) was already in use in the assay system. Although the overall AMLR response in normal rabbits remained relatively low in comparison to that observed in normal human individuals, an increase was apparent upon increasing the FCS concentration, in contrast to the observation of Milthorp and Richter (1979).

In contrast to human PBL, no significant differences in the AMLR were noted with changing cell ratios or cell densities. Indeed, only at low stimulating cell numbers was a decrease apparent in the proliferative response. Optimal cell concentrations were  $2 \times 10^5$  cells/well and  $4 \times 10^5$  cells/well, the optimal stimulating-to responding-cell ratio being 1:1 in both cases. This ratio was also found to be optimal when culturing human PBL in the AMLR.

Despite the attempts to increase the degree of proliferation in the normal rabbit AMLR, the maximal net response attained was 452cpm. The mean normal rabbit response (see Figure 8.4) was 359cpm, approximately 30 fold lower than that observed in the normal human AMLR. Autologous MLR between normal rabbit PBL in the study of Milthorp and Richter (1979) represented the proliferation of whole PBL to mitomycin-C treated whole PBL. The system of Milthorp and Richter (1979), with  $1 \times 10^6$  responding and  $0.5 \times 10^6$  stimulating cells yielded a proliferative response of approximately 600cpm. Taking into account a 10 fold increase in the number of responding cells, and a 5 fold increase in the number of stimulating cells in the Milthorp and Richter study, the proliferative response observed in the normal rabbit AMLR in this thesis is higher.

#### 9.4 The normal rabbit AMLR

The proliferative response of normal rabbit AMLR, despite optimisation, was substantially lower than that observed in the controls for the normal human AMLR. Thus, the authenticity of the observed low response in the normal rabbit AMLR was only ascertained following numerous observations.

Considerable variation was observed in the normal AMLR responses, shown in the 'pre-immunisation state' group of Fig. 8.4, for both the control rabbits and those rabbits in which EAMG was to have been induced. However, the relative range of responses observed in the normal rabbit AMLR (252cpm to 452cpm; mean response 359cpm) was substantially lower than those observed in the normal human AMLR discussed in the previous chapter (381cpm to 56384cpm; mean response 12228cpm).

The range of responses observed for the normal rabbit AMLR (Fig. 8.4, page 210) is more normally distributed than the apparently skewed distribution of the normal human AMLR response (Fig. 4.9, page 104).

Repeat assays of both the rabbit (Figs. 8.7-8.12) and human (section 5.7) AMLR's were consistently reproducible, confirming the different distributions in the two systems.

The kinetic responses observed in the normal rabbit AMLR were similar in profile to those of the normal human AMLR. However, no detectable normal rabbit AMLR response occurred until day 6 in culture, in contrast to the observation of an AMLR after day 3 in culture with normal human lymphocytes.

Whereas very little published data exist on the normal rabbit AMLR, more data have been reported on the mixed lymphocyte reaction (MLR) between the PBL of different rabbits. Difficulties have been reported in obtaining MLR using PBL (Harrison et al., 1971; Knight et al., 1971), although MLR have been described in New Zealand white rabbit PBL's (Sheppard et al., 1977). The MLR reported by Sheppard et al., (1977) were interesting in that they fell into three main categories; high responders (6,000cpm-9,000cpm incorporated/culture), low responders (1,000cpm-3,000cpm incorporated/culture), and non-responders in which no incorporation of  $^{125}\text{I}$ -Uridine into newly synthesised RNA was found. The kinetics of the MLR of the responding rabbits PBL were also similar to those seen in the AMLR cultures of normal human individuals in the present study.

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The cellular mechanisms that account for the observed response in the rabbit AMLR can only be speculated upon here. The low responses observed for the AMLR in this report, and for the MLR noted elsewhere, may reflect a far less interactive cellular immune response in rabbits than that in man. Rabbits are well-known for their ability to generate antibodies and are used as such for research purposes. It may be that the rabbit depends far more than humans on an antibody mediated immune response. If this were the case, then what type of mechanism prevents an anti-antigen response from developing into a pathological anti-self response? The rabbit may depend on mechanisms described in the first chapter (section 1), such as anti-idiotypic responses, immune complexes and blocking factors. Other investigators have noted differences in the immune responsiveness of different populations of the same breed of animal, and population-specific differences cannot be excluded.

Despite the generally low AMLR's of the rabbit system, consistent differences were observed between the responses of normal and EAMG animals. Attempts to augment the normal rabbit AMLR were only partially successful. Augmentation of the normal rabbit AMLR by the use of FCS supplemented medium yielded some success, especially in comparison to the use of autologous rabbit serum. Ponzio (1980) augmented the syngeneic mixed lymphocyte reaction (the murine

equivalent of the AMLR) in a range of inbred mice with 4% polyethylene glycol. However, the use of such chemical augmentation was not considered to be appropriate in this study.

The much lower AMLR response observed in normal rabbits, compared to that of normal humans, prevents any reasonable comparison between the possible immunoregulatory mechanisms that may occur in rabbits and that, speculated on in section 5, in humans. However, the low AMLR proliferative response in rabbits, coupled with the relatively low mitogenic responses, suggests that the rabbit does not possess the level of immunoregulation that humans possess. A humoral form of immunoregulation, possibly utilising an idiotypic network or immune complex formation, or alternatively, a cellular form of immunoregulation may exist in rabbits which was not detectable with the assay systems used in this study. Much of the work carried out on human immunoregulation followed similar studies in the murine system. Indeed, the murine immune system has been suggested as exhibiting a high level of immunoregulation which is capable of complex feedback interactions (Eardley et al, 1978; Gershon et al, 1981; Green et al, 1983). Thus, it is possible that the rabbit immune system may possess an equally potent form of cellular immunoregulation which was undetectable with the assay system employed in this thesis.

### 9.5 Mitogen-induced responses of normal rabbit PBL

Concurrent with the study of the AMLR in normal rabbits, the responses of normal rabbit T cells to the mitogens PHA-P and Con-A were studied. Figure 8.13 shows the scatter of mitogen-induced T cell proliferative responses for normal rabbit PBL as the 'pre-' group of responses. A considerable variation in the Con-A induced responses was observed; the proliferative responses ranging from 13,500cpm to 48,500cpm. This range is approximately half of the range observed when human T lymphocytes were stimulated under the same conditions. However, previous investigators have shown similar Con-A induced proliferative responses. Sheppard et al., (1976) reported normal rabbit Con-A induced T cell proliferative responses of 16,935cpm to 19,757cpm. The same investigators reported normal rabbit PHA-P induced T cell proliferative responses of 5,071cpm to 13,177cpm. The PHA-P induced responses of normal rabbit T cells studied in this thesis ranged from 9,000cpm to 33,989cpm, similar in magnitude to those reported by Sheppard et al., (1976). The PHA-P induced T cell responses of normal human T cells described earlier ranged between 26,204cpm to 158,746cpm, again highlighting the far higher degree of sensitivity to stimulation that human PBL exhibit in comparison to the same types of responses exhibited by rabbit PBL.

The results of lectin-induced proliferation and AMLR responses of rabbit T cells suggest that rabbit PBL are generally much less responsive to stimulatory signals than their human counterparts. This low responsiveness is difficult to explain given the paucity of data that presently exist on the immunological responsiveness of the rabbit.

#### 9.6 The rabbit AMLR in EAMG

Less published data exists for the study of rabbit AMLR in EAMG than was found for the study of the normal rabbit AMLR. No directly comparable data exists for the autologous lymphocytic interactions in rabbits with EAMG observed in this thesis and any other known published material. Thus, the discussion of the rabbit AMLR in EAMG will be limited to a comparison of the AMLR in rabbit EAMG and human MG, and a speculative review of the data presented on the rabbit AMLR in EAMG.

The major difficulty experienced in the assessment of the cellular immune responsiveness in rabbits was the generally low response itself. Although the low normal rabbit AMLR had been established before the induction and maintenance of EAMG in rabbits, it was not known what the result of this disease state would be. The development of an autoaggressive state, where many activated lymphocytes exist in the immune system

and high proliferative responses could occur, was envisaged as one possible scenario. Indeed, the depressed AMLR observed in humans with various autoimmune states may be the result of many years of autoaggression leading to a final state of non-responsiveness, in terms of self-recognition. Additionally, the reports of increased AMLR in certain autoimmune states (Birnbaum and Kotilinek, 1981; Greenberg et al., 1984) may have observed the high self reactivity of patients entering into the initial phases of the autoimmune disease.

The result of the induction of EAMG in rabbits was, however, a decrease in the mean population AMLR response of approximately 30%. The range of these post primary immunisation responses was twice that of the normal rabbit AMLR responses, indicating a high degree of population response variation in the former group. These low responses, maintained in rabbits 58 and 60 but gradually increasing to normal levels in rabbits 57, 59 and 61, may represent the transient blocking or inhibition of certain cell-cell interactions in the AMLR. Only rabbit 62, whose AMLR increased slightly following the first immunisation, showed a substantial decrease in its AMLR following the second immunisation. This was accompanied by a large anti-AChR antibody titre increase, although rabbits 57, 60 and 61 also showed a similarly increased antibody titre following the 'boost' immunisation without an accompanying decrease in their AMLR.

It is possible that the time course of disease maintenance and study was not sufficiently long to monitor any real effects of the disease on the immune system. As is possible in the human case, the long term effects of serum anti-AChR antibody and possibly anti-T cell antibodies, together with activated macrophages may not have been possible to determine. Although initial short-term effects were observed as a decreased AMLR in all the rabbits except rabbit 62, only rabbit 62 showed any signs of a substantially reduced response following the 'boost' immunisation. This observation was also supported by substantially reduced mitogen responses following the 'boost' immunisation. It is possible that anti-T cell antibodies or a breakdown in the activation processes of T cells had occurred in rabbit 62 as a result of the disease induction and maintenance.

The question remains, however, over the usefulness of the AMLR as an indicator of immunoregulatory status in the rabbit in health and disease states. If the assays performed in the study of the rabbit AMLR in this thesis were carried out over longer periods of time, would a clearer picture be generated of the immune system of the rabbit, or is the low AMLR measured in normal rabbits indicative of the use of an assay which is unsuitable for the detection of the rabbit immunoregulatory state. If the rabbit were to be used as the animal model of EAMG for the assessment

of immunomodulatory drugs, it may be more useful to study the changes in specific or non-specific humoral responses than those defining cellular immunoregulation.

#### 9.7 Mitogen responses in EAMG

No differences were observed between the mean population responses of normal rabbit lymphocytes and those of rabbits with EAMG to the mitogens Con-A or PHA-P. The major effects of the immunisations were observed only following several weeks after the initial injection. The greatest effects were seen on the Con-A responsiveness of rabbits 58 and 62; in all other animals both of the mitogen responses remained approximately the same.

Although rabbit 58 rapidly developed clinical EAMG and had to be destroyed, its AMLR response did not correlate with the sudden decrease in Con-A responsiveness, nor with its clinical condition. Thus, it is difficult to reconcile any theories relating to the decrease in Con-A reactivity to any form of cellular control exerted through the AMLR. However, it is possible that the loss of Con-A responsiveness, together with the increase in anti-AChR antibody titre, could indicate the presence of serum anti-T cell subset antibodies. It was suggested in section 1.2.1 that within the Con-A responsive T cell subset existed another T subset which contains T suppressor cells.

Antibodies directed against this subset may lead to the loss of antigen specific suppressor cells and the uncontrolled generation of antigen specific help.

Rabbit 62, however, also exhibited a closely correlatable decreasing Con-A responsiveness with an increasing anti-AChR antibody titre, along with classical clinical symptoms following the booster injection. Approximately four weeks after the onset of changes in the Con-A responsiveness and anti-AChR antibody titre, the AMLR response began to decrease. This may indicate some form of loss of cellular immunoregulation.

It is possible that had rabbit 58 survived past week 10, then a change in its AMLR response may have been observed. However, the earlier initiation of changes in the Con-A responsiveness and anti-AChR antibody titre in rabbit 58 would argue against the possibility that an increased period of study of its AMLR might have revealed any different information.

The Con-A responses of rabbits 57, 59, 60 and 61, whilst showing transient fluctuations, did not exhibit any major trends as shown by rabbits 58 and 62. No appreciable changes were apparent around the mean PHA-P responses of any of the rabbits with EAMG. Thus whilst the cellular responses of rabbits 58 and 62 may have correlated to some extent with the onset of the



clinical disease, as shown by the anti-AChR antibody titre and manifestation of the clinical symptoms, the remaining 2/3 of the diseased population showed no such correlation. Indeed, although clinical signs were apparent in the remaining four rabbits, little or no effect was observed on the cellular immune responses assayed.

## 10.0 CONCLUSIONS AND PROSPECTS

The study of the immunoregulatory changes that may occur prior to, during and following the onset of diseases of the immune system necessitates the use of in vitro assays which are capable of detecting those changes. The AMLR has been reported to be an effective indicator of immune dysfunction in man (Sakane et al, 1978; Gupta 1983; Hafler et al, 1985) and indeed, was shown in this thesis to be defective in patients with the autoimmune disease, myasthenia gravis. It would be extremely difficult to study the changes that may occur in the immunoregulatory mechanisms of man before the onset of the symptomatic disease state. Thus, the rabbit AMLR was studied during the events of the disease process described above in an attempt to elucidate the possible reasons for these changes.

The use of a rabbit system, in which adequate numbers of PBL may be obtained to study the AMLR, was preferred to the use of any other animal e.g. rat, mouse, guinea pig, in which other organ sources of leucocytes would have to be employed. Further, the ability to bleed repeatedly any individual rabbit yields additional advantages to the longitudinal study that was performed. Repeated bleeding of any of the other alternatives would have been at least technically very difficult and even impossible. The use of inbred strains of mice would have yielded a source of syngeneic tissue but may have resulted in other animal

to animal variables. Thus, it was considered that the rabbit represented the best source of tissue for the type of study that was performed.

The difficulties in maintaining EAMG in rabbits, avoiding precipitous death whilst preventing the animal from entering clinical remission, were overcome by the use of a much lower antigen dose regimen. Although the longitudinal study was only carried out over a period of 14 weeks in the remaining four rabbits, clinical symptoms and a high anti-AChR antibody titre were attained following the booster immunisation. It is possible that the clinical condition could have been maintained for an even longer period if further immunisations of low doses of antigen were used.

Overall, the rabbit can be justified as an animal model for the study of AMLR in EAMG because of:

- The ease of obtaining small blood samples at weekly intervals (for serum anti-AChR antibody determinations).
- The ease of the immunisation procedure.
- The obvious signs of the developing clinical condition.
- The ability to obtain adequate samples of PBL repeatedly from the same animal.

The results of the studies on the human AMLR in this thesis suggest a number of possible areas for further study.

A comprehensive study of the normal human AMLR has not yet been achieved and only when the normal response is fully understood can the abnormal response in autoimmune diseases be correctly interpreted. Numerous sections of the normal AMLR require further investigation:

- i) Standardisation of culture conditions and experimental procedures that would enable closer inter-laboratory comparisons.
  - ii) Longitudinal studies of individuals to ascertain the variation within any individuals responsiveness.
  - iii) Classification of the response: can individuals be placed into low, medium or high type response groups?
  - iv) Association of HLA type with the level of the proliferative response.
- .....

Considering the role of immune associated self antigens (HLA-Dr) in the activation process of the AMLR, the degree of association of HLA type with responsiveness may possibly be high. Whilst the study of Davey et al, (1984) suggested an association of HLA-Dr type with AMLR response, further studies may clarify this association. This, together with the increasing amount of data which suggests that many autoimmune diseases show a high degree of association with particular HLA haplotypes, would support the need for an increased effort in this area of investigation. If certain HLA haplotypes predispose an individual to a low degree of immune surveillance, as may result from a low level of lymphocytic interaction as in the case of a low AMLR response, and the individual also carries haplotypes which may predispose to the development of an autoimmune disease (e.g. HLA-B27 and ankylosing spondylitis (Wakefield et al, 1983)), then the triggering of an autoimmune response may proceed in the absence of adequate immunoregulatory control.

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